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Forensic analysis of human DNA from samples contaminated with bioweapons agents

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Contractor's Document Number: 3780-2011-32ey
Contract Project Manager: Della Wilkinson

CSA: Sheldon Dickie, DRDC Centre for Security Science

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Abstract

The purpose of this research project was to further characterize and improve a preliminary protocol for removal of bacterial agents and toxins from samples that would undergo DNA analysis. The protocol must result in samples that are free of infectious or toxic material, but still generate DNA of adequate quantity and quality to meet RCMP standards for identification based on DNA.

In order to obtain and identify possible criminals from DNA evidence, human samples must undergo various steps to isolate and analyse the DNA in a forensic laboratory. These steps consist of (1) the isolation of DNA from samples collected at the crime scene, (2) quantification of the DNA (3) amplification of specific regions on the human chromosomes, (4) analysis of the sequence of amplified DNA, and (5) comparison with possible suspects whose DNA has been banked in the National DNA Data Bank (NDDB) or whose DNA has been collected as part of the investigation.

Résumé

Ce projet de recherche vise à caractériser plus précisément et à améliorer un protocole préliminaire d'élimination des agents bactériens et des toxines dans des échantillons qui feront l'objet d'une analyse d'ADN. Les échantillons obtenus au moyen de ce protocole devraient être exempts de matières infectieuses ou toxiques, mais permettre d'obtenir une qualité et une quantité d'ADN suffisantes pour satisfaire aux normes de la GRC pour l'identification basée sur l'ADN.

Dans le but d'obtenir des preuves basées sur l'ADN et, ainsi, d'identifier d'éventuels criminels, les échantillons humains doivent subir divers procédés visant à isoler et à analyser l'ADN dans un laboratoire judiciaire. Ces procédés comprennent 1) l'isolement de l'ADN à partir d'échantillons recueillis sur le lieu d'un crime; 2) la quantification de l'ADN; 3) l'amplification de régions spécifiques des chromosomes humains; 4) l'analyse de la séquence de l'ADN amplifié; 5) la comparaison avec des suspects possibles dont le profil d'ADN est conservé dans la Banque nationale de données génétiques ou dont l'ADN a été recueilli dans le cadre d'une enquête.

Executive summary

Forensic analysis of human DNA from samples contaminated with bioweapons agents:

Jason Timbers; Kathryn Wright; DRDC CSS CR 2011-22; Defence R&D Canada – CSS; October 2011.

Purpose: The purpose of this research project was to further characterize and improve a preliminary protocol for removal of bacterial agents and toxins from samples that would undergo DNA analysis. The protocol must result in samples that are free of infectious or toxic material, but still generate DNA of adequate quantity and quality to meet RCMP standards for identification based on DNA.

Hypotheses

The main hypothesis of the project is that the standard protocol for extraction of DNA will denature protein toxins and bacteria, and that a protocol previously developed for elimination of infectious bacteria from biological samples during extraction of human DNA is adequate for removal of residual infectious material, including bacterial spores, without compromising human DNA samples.

A second hypothesis is that use of a small robotic instrument in a field situation will provide higher and more consistent yields of DNA than a manual process.

This project can be divided into four objectives:

1. To confirm that the preliminary protocol developed for the removal of infectious bacteria from human biological samples can eliminate or inactivate all infectious material using higher amounts of bacteria than previously tested. As part of these experiments, the infectivity of contaminating bacteria and spores will be assessed at various stages in the DNA extraction process.

2. To determine if the DNA extraction procedure denatures toxins.

3. To compare the quantity and quality of extracted DNA from blood and saliva in the absence and presence of contaminating material using two silica-coated magnetic bead extraction methods: An automated robotic system and a manual extraction of DNA using existing RCMP protocols.

4. To determine if prolonged exposure of biological samples to live bacteria or toxins will affect DNA yield

Sommaire

Analyse médico-légale de l'ADN humain à partir d'échantillons contaminés par des agents utilisés comme armes biologiques Jason Timbers; Kathryn Wright; DRDC CSS CR 2011-22; Defence R&D Canada – CSS; Octobre 2011.

Objet : Ce projet de recherche vise à caractériser plus précisément et à améliorer un protocole préliminaire d'élimination des agents bactériens et des toxines dans des échantillons qui feront l'objet d'une analyse d'ADN. Les échantillons obtenus au moyen de ce protocole devraient être exempts de matières infectieuses ou toxiques, mais permettre d'obtenir une qualité et une quantité d'ADN suffisantes pour satisfaire aux normes de la GRC pour l'identification basée sur l'ADN.

Hypothèses

Selon la première hypothèse de ce projet, le protocole standard d'extraction d'ADN entraînera une dénaturation des toxines protéiques et des bactéries, tandis qu'un protocole précédemment mis au point pour éliminer les bactéries infectieuses dans des échantillons biologiques pendant l'extraction de l'ADN humain convient pour l'élimination des matières infectieuses résiduelles, y compris les spores bactériennes, sans altérer les échantillons d'ADN humain.

Selon la deuxième hypothèse, l'utilisation d'un petit instrument robotique sur le terrain permettra d'obtenir des rendements en ADN plus élevés et plus constants qu'un procédé manuel.

Ce projet comporte quatre objectifs :

1. Confirmer que le protocole préliminaire mis au point pour l'élimination des bactéries infectieuses dans des échantillons biologiques humains peut permettre d'éliminer ou d'inactiver toutes les matières infectieuses, en utilisant des quantités plus grandes de bactéries que lors de tests antérieurs. Au cours de ces expériences, l'infectiosité des bactéries et des spores contaminantes sera évaluée à diverses étapes du procédé d'extraction d'ADN.

2. Déterminer si le procédé d'extraction d'ADN entraîne une dénaturation des toxines.

3. Comparer la quantité et la qualité d'ADN extrait dans le sang et la salive, en l'absence ou en présence de matières contaminantes, en utilisant deux méthodes d'extraction faisant appel à des billes magnétiques enrobées de silice : un système robotique automatisé et un une technique manuelle d'extraction fondée sur les protocoles existants de la GRC.

4. Déterminer si une exposition prolongée des échantillons biologiques à des bactéries ou à des toxines vivantes aura une incidence négative sur le rendement en ADN.

Table of contents

List of figures

List of tables

1 Introduction

In order to obtain and identify possible criminals from DNA evidence, human samples must undergo various steps to isolate and analyse the DNA in a forensic laboratory. These steps consist of (1) the isolation of DNA from samples collected at the crime scene, (2) quantification of the DNA (3) amplification of specific regions on the human chromosomes, (4) analysis of the sequence of amplified DNA, and (5) comparison with possible suspects whose DNA has been banked in the National DNA Data Bank (NDDB) or whose DNA has been collected as part of the investigation.

Contact with a surface (fabric, flooring, soil, etc.), exposure to lighting, temperature and humidity conditions and chemical or biological contaminants are contributing factors that limit the chemical stability of DNA, or that may interfere with DNA isolation and amplification (Kobilinsky, 1992; Sensabaugh & Blake, 1993). Dirt, fire debris, microorganisms and DNA from innocent bystanders are but a few of the contaminating factors found in crime scenes. Biological evidence may also come in contact with various chemicals that have effects on human DNA. Organic solvents such as alcohol, gasoline and cleaning fluids seem to cause little or no damage to DNA, while acids and alkalis generate chemical modification of DNA bases. Formaldehyde gas, detergents, bleach and hydrogen peroxide vapour that may be used to decontaminate crime scenes can damage DNA (Kobilinsky, 1992; Sensabaugh & Blake, 1993).

Even when intact, some DNA is refractory to amplification where the inhibition is due to the presence of polymerase inhibitors. Interfering inhibitors can be found in pigments of blood, such as free heme, in tar in cigarette butts and dye in clothing (Sensabaugh & Blake, 1993). Commercially available kits for DNA isolation now include reagents that counteract such inhibitors (Sensabaugh & Blake, 1993).

It is known that microbial contamination with *Staphylococcus epidermis, Candida valida, Escherichia coli and Bacillus subtilis* can induce strand scission in human DNA as a result of microbial nucleases, but such samples may still provide acceptable DNA yields and profiles if human specific primers are used (Fattorini *et al*, 2000; Kobilinsky, 1992; Sensabaugh & Blake, 1993). Additionally, when microorganisms are present, the eluted mixture will contain both human and microbial DNA, and so the human DNA profile could be misinterpreted. Primers designed to amplify specifically human DNA loci should function even in the presence of microorganisms in the samples, and so should generate suitable yields of amplified human DNA for analysis (Kupfer *et al*, 1999, Fernandez-Rodriguez et al, 1996; Webb et al, 1993). However, amplification inhibition or generation of allele dropouts have been seen with the co-presence of microorganisms, such as the common vaginal microorganisms *Candida albicans, Gardneralla vaginalis* and *Bacteroides fragilis* (Lienert & Fowler, 1992). And, it was shown in one study that microbial DNA could be the source of extra bands observed when typing of a specific human locus, D1S80, was carried out with forensic biological samples contaminated with some microorganisms, including Corynebacterium sp., *Streptococcus sanguis, Micrococcus luteus, Klebsiella pneumoniae* and *Pseudomona stutzeri* (Fernandez-Rodriguez *et al*, 1996). Other organisms tested had no effect.

In addition to the issue of DNA degradation or inhibition of DNA amplification due to the presence of contaminating microorganisms, investigators must also be concerned with the potential dangers of contaminating materials. There are no standard decontamination procedures established to deal with crime scenes where microbes and other biological weapons might exist. When a site is known to contain potentially deadly biological agents, decontamination of the entire area seems to be the common choice for rapidly removing potential danger for the investigating teams and the population. Chemicals, such as formaldehyde gas, detergents, bleach and hydrogen peroxide vapour (HPV), are effective at reducing infectivity of many microbes, but these reagents also degrade DNA (Hoile *et al*, 2010). Another technique used to remove infectious threats from a crime scene would be to irradiate the entire area, though this method may miss well-concealed biological agents. However, movement of the materials from the contaminated area into a safe zone, such as a mobile laboratory, prior to treatment would guarantee direct contact to irradiation. Bacterial spores are the most resistant of biological agents, and the quantity of gamma rays to eradicate the infectivity of spores may also reduce the quantity of DNA recovered (Hoile *et al*, 2010). The application of irradiation on buccal cells has been shown to reduce the yield and quality of DNA by compromising longer DNA fragments, rendering the amplification of the whole-genome unfeasible, but a partial analysis possible (Castle *et al*, 2003).

The approach of retrieving human biological samples from a crime scene and then decontaminating them prior or during analysis may be viable using other methods of decontamination. Apart from the previously discussed chemical and irradiation methods, there is also physical decontamination. Physical methods consist of thermal decontamination, filtration or separation of contaminated samples. When samples are to be collected for isolation of human DNA, thermal decontamination involving heat inactivation of the biological agent is not practical since boiling or autoclaving will destroy human DNA. Another option is filtration or separation, which is unable to inactivate the biological agents, but would retain larger agents, such as bacteria and spores, although viruses and toxins would pass through.

Work previously carried about by the RCMP in collaboration with Public Health Agency of Canada (PHAC) tested such a method for decontamination of human DNA extracted from blood or saliva (Hause, 2007). In a total of 255 blood and saliva samples seeded with *Francisella tularensis*, *Yersinia pestis* or spores of *Bacillus anthracis*, it was shown that the use of temperatures as high as 95ºC for thirty minutes could not guarantee complete inactivation of contaminating bacteria or spores, but filtration of samples through a 0.22µm filter at the end of the purification process totally eliminated any residual infectivity of the contaminating agents without compromising the quantity and quality of human DNA. Filtration of samples before DNA extraction was also effective at removing infectivity of the selected pathogens but there was some reduction in the yields of DNA, and it was suggested that this procedure could increase the possible cross-contamination of samples with DNA from the technician during the manipulation (Hause, 2007). However, delaying the removal of the agents prior to DNA extraction means that personnel may be at risk of exposure during the extraction process.

Many aspects of this proposed procedure were not analysed in detail. As noted, doing the filtration step at the end of the protocol means that samples must be considered infectious or toxic throughout the process. It would be important then to know if infectivity of contaminating bacteria and spores, or integrity of toxins, could be reduced or lost at earlier stages in the DNA extraction process. The proposed protocol was tested on relatively small numbers of

bacteria/spores, so we do not know if the protocol will eliminate infectivity when higher numbers of microbes or bacterial spores are present in samples. The protocol has also not been tested to determine if other types of potential biological weapons such as toxins, will be degraded, nor do we know the effect of toxins on human DNA integrity. The effects of prolonged exposure to biological weapons on human DNA were not tested. Lastly, it would be of interest to determine if an automated DNA extraction process that would be appropriate for use at a crime scene could be incorporated into the protocol. Such a system would theoretically reduce risk of exposure of personnel to potentially dangerous agents by limiting manual manipulation of samples, but could also improve yields and quality of isolated DNA.

Purpose:

The purpose of this research project was to further characterize and improve a preliminary protocol for removal of bacterial agents and toxins from samples that would undergo DNA analysis. The protocol must result in samples that are free of infectious or toxic material, but still generate DNA of adequate quantity and quality to meet RCMP standards for identification based on DNA.

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- To determine if the DNA extraction procedure denatures toxins.
- To compare the quantity and quality of extracted DNA from blood and saliva in the absence and presence of contaminating material using two silica-coated magnetic bead extraction methods: An automated robotic system and a manual extraction of DNA using existing RCMP protocols.
- To determine if prolonged exposure of biological samples to live bacteria or toxins will affect DNA yield.

2 Materials and Methods

Bacteria: Frozen stocks of *Pseudomonas aeruginosa* (ATCC 15442) and *Staphylococcus aureus* (ATCC 6538) were received from Dr. S. Sattar, University of Ottawa. *Yersinia pseudotuberculosis* was purchased from the American Type Culture Collection (ATCC 11960). Spores from the avirulent *Bacillus anthracis* Sterne strain (34F2) were kindly provided by Denis Laframboise at the Public Health Agency of Canada (PHAC, Ottawa), while a virulent strain of *Bacillus anthracis* (bovine) (NML 03-0191) was obtained from the National Microbiology Lab (Winnipeg). To prepare stocks of *Pseudomonas* and *Staphylococcus* bacteria, 1mL of thawed bacteria was added to 100mL of tryptic soy broth (TSB) as per the manufacturer's recipe (30g/L) (BD Difco™). Cultures were incubated on a shaker overnight at 37ºC, and stocks were aliquoted and stored at 4ºC. The entire pellet of *Y. pseudotuberculosis* was rehydrated in 1mL of TSB, then transferred into 5mL of TSB, thoroughly mixed, and several drops were inoculated onto tryptic soy agar (TSA) plates (40g/L, BD Difco). Plates were incubated at 37ºC for 48 hours and then colonies were inoculated into broth, incubated, aliquoted and stored in the same fashion as described above. To rapidly determine the concentration of bacteria in subsequent cultures of these bacteria, a standard curve of absorbance at a wavelength of 600nm was graphed against the log of colony forming units (CFU) for each bacterium for each hour over a 24 hour period. These graphs were used in subsequent experiments to determine the quantity of bacteria added to the samples.

To generate stocks of spores from *B. anthracis*, a protocol from NML was followed. Briefly, *B anthracis* bacteria (NML 03-0083) were inoculated into a 250mL flask containing 1:10 Columbia broth-$MnSO_4$. Media was prepared from powdered media (BD Difco™), at 35g/L in sterile water where manganese sulphate ($MnSO_4$) was added to yield 0.1mM. The flask was incubated at 37ºC for 72 hours on an orbital shaker at 150-200 cycles/minute. The bacteria were pelletted by centrifugation and washed three times with 50mL of sterile water. After the last centrifugation, the pellet was resuspended in 10ml of sterile water. Vegetative bacteria were heat inactivated by incubation at 80°C for 10 minutes. Dilutions of the remaining solution of spores were plated on TSA and incubated overnight to determine the spore counts (1 colony = 1 spore). The stock was adjusted to a concentration of 10^9 CFU/ml, and aliquots were stored at 4°C. For

experiments conducted at PHAC, stocks of spores had been prepared by PHAC personnel and were adjusted to the appropriate concentration prior to experiments

All work with infectious *B. anthracis* was carried out in containment level 3 laboratories (CL3) at the PHAC or at the University of Ottawa. At the end of the experiments, all DNA samples were incubated for one week on TSA to ensure no residual infectivity prior to removal from the lab for further analysis. To exit the CL3, DNA sample tubes were wrapped in paper towel and soaked with bleach (1:10) for ten minutes. DNA sample tubes were then placed in a sealed box for removal as specified by CL3 standard operation procedures (SOP).

Toxins: Botulinum toxin A (Sigma B8776) and Staphylococcal enterotoxin B (Sigma S4881) were purchased from Sigma-Aldrich Canada Ltd. (Oakville). Both toxins were hydrated with sterile water to yield stocks of 1mg/mL. The toxin solutions were stored at 4ºC. Rather than work with ricin, a toxoid was kindly provided by Cangene (Winnipeg). This toxoid (TST 10088) is a modified form of ricin which has lost its toxicity due to the addition of an extra covalent linker between the two chains of the toxin. The stock received from Cangene (0.86mg/mL) was diluted to a concentration of 3µg/mL and stored at 4ºC.

SDS-PAGE: For electrophoresis of proteins, 12.5% Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as per Laemmli (Laemmli, 1970). Samples were mixed with 2X Laemmli sample buffer (0.125M of Tris, pH6.8, 4% SDS, 10% β-mercaptoethanol, 20% glycerol, and 0.004% bromophenol blue) and boiled for a minute prior to loading. Gels were fixed (5:4:1, H_2O-MeOH-acetic acid) and stained with Coomassie blue (one tablet of R250 was diluted in 1L of fixative solution) (Fisher Scientific). Gels were destained, as necessary, in a mixture of water, glacial acetic acid and methanol (7:1:2). Molecular weight markers were included in every gel (PageRuler™ Prestained Protein Ladder Plus, Fermentas Canada Inc.).

ELISA: ELISA assays were performed with commercially available kits for the detection of botulinum toxin and ricin (BioThreat Alert® ELISA kits, Tetracore®). Briefly, plates were incubated with blocking buffer overnight at 37ºC (5% skim milk powder in phosphate buffered saline with 0.1% Tween-20, PBST) and washed 4X with PBST prior to adding varying dilutions

of samples to be tested. A standard curve of the toxin alone and toxin mixed with blood was included in all assays, as were negative controls of blocking buffer alone with all other reagents. Plates were incubated at 37ºC for an hour in the dark and then washed four times with PBST. The conjugated antibody was added as per the manufacturer's directions. Plates were then incubated for another hour at 37ºC. Finally, the peroxidase substrate (100μL) was freshly mixed and added to each well. After a final incubation at 37ºC for thirty minutes, plates were read by an ELISA microplate reader at a wavelength of 405nm.

DNA isolation from human samples: Before each experiment, working surfaces were decontaminated with 70% Ethanol. Samples were manipulated with sterile forceps which were decontaminated in 80% Ethanol before and after each use, to prevent cross-contamination. While working with DNA samples, a surgical mask and latex gloves were worn to prevent any contamination of the samples with self DNA. Blood from two anonymous donors at the RCMP was generously provided in anti-coagulant (5.4mg of K_2EDTA) for each set of experiments. Blood was refrigerated at 4°C and used for a maximum period of 1 week. Prior to use, the blood was shaken for 15 minutes, then diluted in PBS to generate samples of 10μL containing 0.1 to 10μL of blood. Samples were transferred to sterile swabs and left to air-dry on a surface free of human DNA for an hour. Epithelial cells contained in the saliva from the inside cheeks of a donor were collected via buccal swab. Buccal swabs were similarly air-dried for an hour. Bacteria, spores or toxins were added in various amounts to the swab containing air-dried blood and saliva. The volume of contaminating material ranged from 10 to 100 μL. Following the application, the contaminated swabs were left to dry and then incubated for varying times prior to isolation of human DNA. Control samples for each set of experiments were prepared simultaneously as the blood and saliva samples. For negative controls to ensure that bacterial DNA would not be detected, three swabs containing the relevant biological agent without any trace of human DNA were prepared. Positive controls were whole blood samples (10μL) on swabs without any added biological agents, also done in triplicate. For negative controls for the later PCR steps, three empty sterile sample tubes and three blank swabs dipped in PBS were included. Once samples and controls were ready for extraction, they were placed in sterile 2mL screw-cap tubes (EZ1 DNA Investigation kit, Qiagen) and refrigerated at 4°C until extraction, except for the time-exposure experimentation samples, which were incubated at room temperature for various periods up to one week.

DNA was extracted from samples using commercially available kits with some modifications. Samples on swabs were incubated overnight at 56^{o}C in 300 uL of an RCMP lysis buffer (REB, 100mN NaCL, 10mM Tris HCL, pH 8.0, 10mM Ethylenediaminetetraacetic acid, EDTA, 0.5% sarkosyl and 40mM dithiothreitol, DTT) to which Proteinase K was added to make a final concentration of 10mg/mL (Qiagen). Swabs were then placed in a spin basket (Spin-eZe™ baskets, Fitzco Inc) and centrifuged at 10,000xg for 5 minutes in order to remove excess liquid. The spin basket and swab were discarded and carrier RNA (cRNA, Qiagen) was added to all samples at a final concentration of 1µg/µL. In some experiments, samples were split into two fractions of approximately 150µL prior to the next step.

Manual extraction was carried out using the DNA IQ™ kit (Promega). DNA IQ™ resin (12µL/sample) was added to the samples. The tubes were vortexed to mix the resin and the DNA and left at room temperature for at least five minutes. DNA IQ Lysis Buffer (700µL/sample) was then added, and samples were incubated at room temperature for fifteen minutes while being mixed by vortex every minute. After incubation, the sample tubes were pulsed in a centrifuge for twenty seconds, then placed on a magnetic separator stand to pellet the resin. The resin was twice washed with DNA IQ Magnetic Bead Wash (200µL/sample) and the solution was spun, vortexed and returned to the magnetic separator stand for ninety seconds. The washes were carefully discarded without disturbing the resin. The tubes were left for ten minutes at room temperature with the lids open to allow for residual wash solution to evaporate prior to the addition of the Elution Buffer provide with the kit (60µL). The samples were vortexed, spun and incubated at 65°C for 8 minutes twice before collecting the eluted DNA, which was transferred to a clean 1.5mL sterile tube.

Automated DNA extractions were carried out with robot EZ1® Advanced [Qiagen]. Cartridges containing all necessary reagents (i.e. lysis buffer, elution buffer, resin, etc.), filtered tips and tip holders were provided with the EZ1® DNA Investigation Kits. These were placed into the EZ1® Advanced workstation along with the sample tubes. The DNA purification program automatically proceeded for an approximate time of twenty minutes, resulting in a final volume of 100µL of DNA from each sample in TE buffer. After the removal of the DNA from the

instrument it was cleaned with 70% Ethanol and decontaminated with a twenty minute ultraviolet light sweep.

In experiments where manual and automated extracted samples were filtered as a final step, the DNA sample was passed through a low-binding Durapore® PVDF 0.22µm membrane by centrifugation as specified by the manufacturer (Ultrafree®-MC centrifugal filter, Millipore). Half of the filtered samples (50µL) were tested for the presence of contamination with bacteria, spores or toxins. The rest of the samples were stored at -20 °C until DNA identification analysis.

DNA analysis: DNA quantification, PCR amplification, sequencing and capillary electrophoresis were carried out at the RCMP by Jason Timbers. DNA was quantified using the *Quantifiler® Human DNA Quantification* (Applied Biosystems). This kit detects only human DNA in samples. Briefly, the *Quantifiler® Human DNA Quantification* reaction mix (12.5µL) and provided primers (10µL) were placed into the wells of a 96-well plate. Extracted DNA samples (2.5µL) were added to their respective wells to result in a total volume of 25µL in each well. A standard curve using control DNA supplied by the RCMP was included on every plate. If quantification was not carried out immediately, plates were stored at room temperature in the dark after being sealed with MicroAmp® Optical Adhesive Film (PCR compatible, DNA/RNA/RNase Free, Applied Biosystems). Before proceeding, plates were centrifuged at 4,000 rpm for 4 minutes to remove excess condensation and bubbles in the wells. The plate was then inserted into the 7500 RT-PCR system (Applied Biosystems) which provides fluorescent readouts of amplified products from human DNA in the sample, and quantification of starting amounts of DNA in samples are determined based on the standard curve.

To proceed to the next step in analysis, the quantity of DNA should be higher than 0.250ng. If necessary, samples were concentrated using Montage™ PCR Centrifugal Filter Devices (Millipore). Samples were diluted or concentrated so that each well contained 0.5ng of DNA. To detect the pattern of Single Tandem Repeats (STR) in the DNA samples, the AmpF*l*STR™ Profiler Plus kit was used (Applied Biosystems). The supplied PCR Reaction Mix (5.7µL), Profiler Plus Primer Set (3.0µL) and AmpliTaq Gold™ DNA Polymerase (0.3µL) were added to each well as recommended by the manufacturer. For each sample, 6µL of DNA was added. Controls of wells that were negative (FAD water) and positive (standard DNA from the

Profiler Plus Kit) were included on each plate. MicroAmp™ Amplification Adhesive Film [Applied Biosystems, *Carlsbad, California, USA*] was secured over the wells and the plates were placed in PCR thermo cycler (MJ Research PTC-200, [*St. Bruno, Québec, Canada*] or Bio-Rad C1000, [*Mississauga, Ontario, Canada*]) and covered with a jelly mat. Overnight amplification was completed using the following cycling parameters: 95ºC, 11 minutes followed with 28 cycles of denaturation for 60 seconds at 94ºC, annealing of primers for 90 seconds at 59ºC and extension for 90 seconds at 72ºC. A final extension at 60ºC for 45 minutes followed by an overnight incubation at room temperature was also included. The 96-well plates were stored at -20°C until required for analysis by capillary electrophoresis.

Amplified products were prepared for analysis by capillary electrophoresis (CE) using the Freedom EVO® liquid handling system (Tecan Group Ltd., *Männedorf, Switzerland*). Samples were centrifuged at 4,000 rpm for 5 minutes. HiDi Formamide (20µL) and GeneScan™ 500 ROX™ (0.5µL) (Applied Biosystems, *Carlsbad, California, USA*) reagents were loaded into each well of a 96-well CE plate and 0.25µL of appropriate sample was added. Profiler Plus Ladders (0.75µL) were included on each plate. Prior to each run the tips of the liquid handling system were cleaned with 70% ethyl alcohol and a pipetting test was performed to ensure that the instrument was dispensing accurately. CE plates were heated at 95°C for 5 minutes and cooled at 4°C for 3 minutes, centrifuged, then loaded onto the CE 3130X Genetic Analyses instrument (Applied Biosystems, *Carlsbad, California, USA*) following RCMP procedures to analyse the sizes of the amplified products. After each run, the instrument tips were cleaned with bleach solution (2%).

Analysis of CE results: Results were interpreted by RCMP analysts using ABI PRISM® Gentotyper® software. The 500 ROX DNA ladders were used as a guide for the detection of the amplified STR alleles. To ensure that the data were properly analysed, the analysts verified the Profiler Plus Ladder dye labels (5-FAM, JOE, and NED NHS-ester) to correspond to the Applied Biosystem's ladder templates. The electropherogram produced by the CE was analysed to determine the number of repeats on the selected STRs from the amplified human DNA. Ten loci on various regions of human chromosomes are included in analysis at the RCMP; D3S1358, vWa, FGA, Amelogenin (X & Y), D8S1179, D21S11, D18S51, D5S818 and D13S317 (**Table 2**). The presence of X and Y chromosomes was determined to ensure the appropriate donor

corresponded to the sample type. Degradation of DNA was assessed by dividing the area of the peak (homozygous) or peak averages (heterozygous) of the shortest STR locus (D3S1358) to the largest STR locus (D18S51). A positive DNA control, provided in the AmpF*l*STR™ Profiler Plus kit Applied Biosystems, [*Carlsbad, California, USA*], and a negative control were present for each analysed CE plate.

Statistical Analysis: For each experiment, various volumes of blood (0.1µl, 0.5µL and 1.0µL) from two donors were run in parallel, each divided into non-contaminated and contaminated samples. The entire set was run in duplicate. Saliva was taken from only one donor and was set up similarly as for blood: Buccal swabs with unknown volumes of saliva were collected for contaminated and non-contaminated samples and were prepared in quadruplicate rather than in duplicate. Every experiment was carried out at least three times. Results from all three experiments were pooled for statistical analysis, perform with Microsoft Office Excel 2007 software. Standard derivations (SD) were calculated for each set of extraction samples. A two tail paired Student T-test was performed to compare results from the different extraction methods. If the p value was below 0.05, the variations between samples were considered significant.

3 Results

Due to difficulties in acquiring bacteria that could serve as biological weapons, we completed some experiments with risk level 2 bacteria; *P. aeruginosa*, as a representative gram negative bacteria, and *S. aureus* as a representative gram positive bacteria. As a surrogate for *Y. pestis*, we used *Y. pseudotuberculosis*, which is closely related to *Y. pestis* but lacks a plasmid carrying virulence factors (Zhou and Yang, 2009). For experiments examining spores, we used two strains of *B. anthracis*, one attenuated (Sterne) and one virulent (isolated at NML from a cow spleen).

Objective One: Confirmation of loss of infectivity

The first objective was to confirm that the preliminary protocol developed for the removal of infectious bacteria from human biological samples by using a 0.22μm filter after extraction of DNA can eliminate or inactivate all infectious material using higher amounts of bacteria than previously tested. A general overview of this protocol is shown in Figure 1. Also, in the preliminary work, it was not established whether the filter was really the cause of the removal of the selected bacteria, *B. subtilis*, *S. aureus*, *P. aeruginosa*, *Y. pestis* and *F. tularensis*, and spores of *B. anthracis* (Hause, 2007).

The various steps in the DNA extraction process are designed to denature and eliminate proteins. Hence, it seemed likely that infectivity of bacteria would be lost during the first step in DNA extraction, which is incubation in a lysis buffer (REB) that ruptures cell membranes. Generally such a buffer contains both detergent and proteinases. To test that infectivity is lost after this step, blood samples were placed onto three swabs for each stage of the extraction procedure – after incubation in lysis buffer O/N at 56^{o}C (RCMP lysis buffer with Proteinase K, see Materials and Methods), after full DNA extraction, after DNA extraction and passage through a 0.22 um filter, plus samples representing heat treatment alone. Immediately after an hour of air-drying, 10^{9} bacteria were added to each swab and were air dried for 60 minutes. After the appropriate step, each set of samples was plated on agar plates and assessed for growth after 24 hours. As can be seen in Table 1, incubation at 56^{o}C in lysis buffer totally eliminated infectivity of all three bacteria.

While bacteria were readily inactivated during the first step of the extraction process, spores were expected to be more resistant, based on the preliminary work by Hause (2007) and by

what is known of the resistance of spores to environmental extremes (Ryan and Ray, 2010). In two experiments, 10^9 spores from two strains of *B. anthracis* were applied to human biological samples as described above, and again samples were assayed for infectious spores at various stages during DNA extraction. In Table 2, it can be seen that the number of infectious spores is only minimally reduced after incubation in the lysis buffer, and more markedly reduced by the end of the extraction process. However, 3 to 4 spores can still initiate infection (Peters and Hartley, 2002), and hence the filtration step is required to ensure safety of individuals carrying out the complete analysis of such samples.

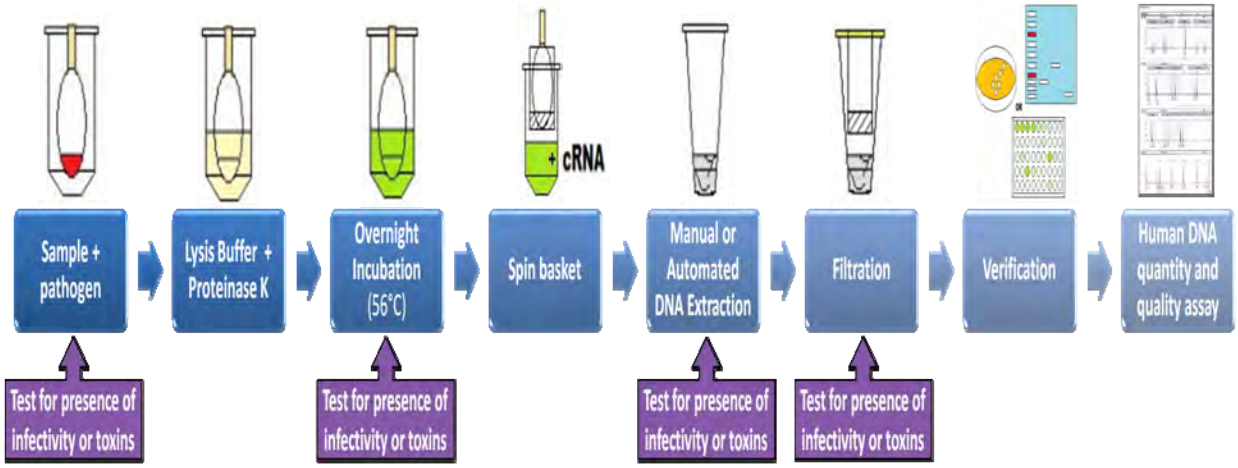

Figure 1: Overview of DNA extraction process

Conclusions:

These experiments show that the protocol developed previously is effective at eliminating infectivity of both vegetative bacteria and spores. Infectious vegetative bacteria were inactivated after the first incubation step during DNA extraction, even with the presence of as many as 10^9 bacteria. Spores were resistant to the extraction process, and thus samples with unknown contaminants must be considered to be infectious until the final filtration step has been completed. The final filtration will also remove any bacteria that might have escaped inactivation to this point.

Table2 Colony forming units of bacteria at various stages of the DNA extraction process

Treatment	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Yersinia pseudotuberculosis*
Positive growth control	150+ /150+ / 150+	150+ / 150+ / 150+	150+ / 150+ / 150+
After O/N freeze (aliquot)	150+ / 150+ / 150+	150+ / 150+ /150+	150+ / 150+ / 150+
Incubation at 56°C	0 / 0 / 0	0 / 0 / 0	4 / 0 / 0
Cell lysis at 56°C	0 / 0 / 0	0 / 0 / 0	0 / 0 / 0
DNA capture	0 / 0 / 0	0 / 0 / 0	0 / 0 / 0
After filtration	0 / 0 / 0	0 / 0 / 0	0 / 0 / 0
Tampered filter	145 / 133 / 139	142 / 142 / 150+	150+ / 146 / 150+

Triplicate samples of 1 uL of blood on cotton swabs were seeded with 10^9 CFU of bacteria, dried, then incubated as indicated in column one. Samples were plated on TSA, and assessed for colonies after incubation overnight.

Table 3 Fate of bacterial spores during the DNA extraction process

	Bacillus anthracis Sterne Strain	*Bacillus anthracis NML 03-0191*
Positive growth control	≥150 / ≥150 / ≥150	≥150 / ≥150 / ≥150
After O/N freeze (aliquot)	ND	≥150 / ≥150 / ≥150
Incubation at 56°C	ND	124 / 109 / 131
Cell lysis at 56°C	45 / 34 / 37	31 / 42 / 32
DNA capture	4 / 4 / 3	3 / 4 / 3
After filtration	0 / 0 / 0	0 / 0 / 0
Tampered filter	ND	≥150 / ≥150 / ≥150

Triplicate samples of 1 uL of blood on cotton swabs were seeded with 10^9 spores, dried, then incubated as indicated in colomn one. Samples were plated on TSA, and assessed for colonies after incubation overnight.

Objective Two: Fate of toxins during DNA extraction

Toxins from various sources represent another group of potential biological weapons. Toxins are small molecule poisons, such as lipids, peptides or proteins, produced by living organisms. Therefore, the filtration step will be unable to trap toxins. However, as toxins are mostly proteinaceous in nature, the application of detergent and proteinase K in the lysis buffer during the first incubation step should inactivate or degrade toxins prior to extraction. As for the previous experiments, blood samples were set up for each step in the extraction process, and immediately after an hour of air-drying, toxins were added to the swabs, and dried for another hour. A range of concentrations of each toxin was used in each experiment. The presence of the toxin was assayed at various steps throughout the extraction procedure. Three toxins were chosen for testing; Botulinum toxin A (BoTN/A), Staphylococcal Enterotoxin B (SEB), and ricin.

The LD_{50} for BoTN/A is estimated to be 0.001 mcg/kg by inhalation, and 1 mcg/kg orally. Thus, a lethal dose for a 70 kg human would be about 70 ng inhaled (Arnon et al. 2001). The amount of toxin seeded into samples was 0.01 to 50.0 mcg. BoTN/A in samples was detected using a commercially available capture ELISA kit as described in Material and Methods. On the plates provided, alternate rows of wells are coated or uncoated with a toxin specific antibody to capture toxin in the samples, which is then detected with a second antibody specific for the toxin, but which is enzyme tagged. The difference between the OD values in wells with and without capture antibody confirms the specificity of the reactivity, and this can be seen for the standard curve of toxin alone in Table 3. OD values reach background levels when there is still ~200 ng of toxin present. This is considerably above the sensitivity reported by the manufacturer, which is 1 ng of toxin (Tetracore). Once mixed with blood, the positive reactivity observed in the samples was no longer dependent on the presence of the capture antibody. In other words, the positive reactions in the samples mixed with blood were due to high amounts of total protein, rather than to the specific presence of the toxin (Standard curve, Toxin in blood in Table 3). While the standard curve showed no toxin-specific reactivity, blood samples seeded with toxin and assayed after incubation at 56^{o}C overnight the presence of lysis buffer were positive for the presence of toxin (Figure 2, Lysis and Table 3, Cell Lysis Buffer), while samples incubated at 56^{o}C without lysis buffer displayed no toxin specific reactivity ((Heat Treatment, Table 3; green line Figure 2). Samples that had undergone the complete extraction process after seeding with 1 mcg of toxin displayed a significant loss of general reactivity, representing a

reduction in the amount of total protein as expected during isolation of nucleic acids. There was no toxin specific reactivity when values were corrected for the background (DNA capture in Table 3). Thus, it appears that toxin has been degraded in these samples.

Given the absence of specificity and low sensitivity of this ELISA in our hands, it is difficult to interpret these results. We know that there is toxin present in samples for the "Standard Curve, Toxin in Blood" samples, and yet the assay did not detect this. So there is the possibility that toxin is present in other samples scoring as negative in the assay. On the other hand, incubation of samples in lysis buffer plus proteinase K did reduce the total positive reactivity of all samples, even when samples did not appear to be positive for toxin. While the presence of DTT does not reduce the toxicity of BoNT/A (Cai et al. 1999), there are no indications in the literature that BoNT/A is resistant to the other protein denaturing elements in the RCMP lysis buffer, such as the ionic detergent sarkosyl, and the proteinase K.

Table 3: OD values from ELISA assay for BoNT/A in the presence and absence of toxin-specific capture antibody.

	1	2	3	4	5	6	7	8	9	10	11	12
Toxin added (mcg)	**50.0**	**25.0**	**12.5**	**6.25**	**3.13**	**1.56**	**0.78**	**0.39**	**0.2**	**0.1**	**0.05**	**Blank**
Capture Antibody	**Standard Curve (Toxin alone)**											
+	0,320	0,318	0,340	0,32	0,266	0,296	0,260	0,153	0,117	0,094	0,069	0,054
-	0,198	0,184	0,162	0,15	0,233	0,164	0,112	0,078	0,153	0,096	0,067	0,053
Difference	**0.122**	**0.134**	**0.178**	**0.170**	**0.033**	**0.132**	**0.148**	**0.075**	**0**	**0**	**0.002**	**0.001**
	Standard Curve (Toxin in blood)											
+	0,274	0,287	0,261	0,281	0,249	0,207	0,178	0,142	0,118	0,110	0,060	0,021
-	0,369	0,436	0,346	0,394	0,500	0,389	0,383	0,385	0,145	0,131	0,059	0,027
Difference	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0.01**	**0**
Toxin	**50.0**	**25.0**	**12.5**	**6.25**	**3.13**	**1.56**	**50.0**	**25.00**	**12.5**	**6.25**	**3.13**	**1.56**

added (mcg)	0	0	0				0		0			
	Heat Treatment.						Cell Lysis Buffer					
+	0,243	0,297	0,246	0,216	0,241	0,259	0,144	0,179	0,140	0,080	0,030	0,000
-	0,225	0,301	0,238	0,190	0,232	0,338	0,128	0,118	0,114	0,080	0,010	0,000
	0	**0**	**0.008**	**0.026**	**0.009**	**0**	***0.016***	***0.061***	***0.026***	**0**	**0.020**	**0**
	DNA Capture- 1mcg toxin added						Negative controls					
+	0,026	0,027	0,013	0,014	0,064	0,055	0,083	0,011	0,074	0,065	0,021	0,011
-	0,088	0,077	0,014	0,012	0,061	0,059	0,081	0,011	0,072	0,098	0,021	0,013
	0	**0**	**0**	**0.002**	**0.003**	**0**	**0.002**	**0**	**0.002**	**0**	**0**	**0**

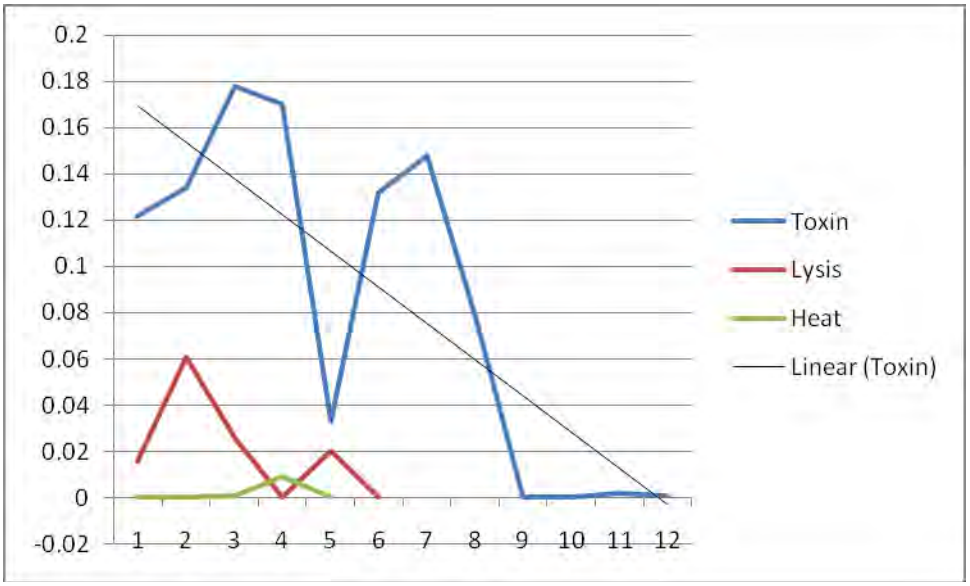

Figure 2. Reduction in Botulinum toxin A at various stages in DNA extraction as determined by ELISA. Samples 1 to 12: toxin added in doubling dilutions from 50 mcg to 0.05 mcg. **Toxin**: standard curve after correction for non-specific reactivity, with linear best fit estimated. **Heat** : O/N incubation at 56°C. **Lysis**: incubation at 56°C in lysis buffer with proteinase K. OD values have been adjusted for background reactivity in the absence of toxin-specific capture antibody.

As acquisition of ricin is highly restricted, a ricin toxoid, kindly provided by Cangene, was used instead. The LD_{50} of ricin is ~3 mcg/kg for injected or inhaled ricin, but much higher for oral ingestion (Schep et al. 2009). Standard curves were prepared with amounts of toxoid ranging from 0.1 to 100 mcg, while test samples were seeded with amounts of ricin toxoid ranging from 3.1 to 100 mcg. The toxoid was also detected using a commercial ELISA kit. In this case positive reactivity in the standards was specific to the presence of toxoid even when mixed with blood, although the toxin specific reactivity was reduced in this case (Table 4, Figure 3). Heat treatment without or with lysis buffer reduced the OD values of blood samples seeded with toxoid, but the latter was more effective. According to the standard curve of toxoid in blood (Figure 3), none of the samples incubated in lysis buffer contained more than 100 ng of toxoid, which is below a toxic dose for the average adult. Samples that had undergone the complete DNA extraction process showed no reactivity over background values (mean OD for 6 samples was 0.183, DNA Capture in Table 4, compared to a mean OD of 0.26 for negative wells). Although not directly quantitative, it is clear that the first incubation step in the DNA isolation process does reduce the amount of toxoid, and presumably of ricin also, to a point that is safe, even when high amounts are present in the starting material.

Table 4: OD values from ELISA assay for ricin toxoid in the presence and absence of toxin-specific capture antibody.

	1	2	3	4	5	6	7	8	9	10	11	12
Amt added (mcg)	**100.**	**50.0**	**25.0**	**12.5**	**6.3**	**3.1**	**1.6**	**0.8**	**0.4**	**0.2**	**0.1**	**blank**
	Standard Curve (Toxoid alone)											
+	2,64	2,66	2,590	2,530	2,860	2,570	2,330	2,480	1,600	0,790	0,630	0,422
-	0,75	0,73	0,870	0,990	0,470	0,440	1,000	1,470	1,450	0,630	0,080	0,406
Difference	**1.89**	**1.93**	**1.72**	**1.54**	**2.39**	**2.13**	**1.33**	**1.010**	**0.150**	**0.16**	**0.55**	**0.016**
	Standard Curve (Toxoid with blood)											
+	2,41	3,04	2,690	2,600	2,620	2,200	2,040	2,750	2,080	1,310	0,570	0,300
-	1,55	1,45	1,350	1,520	1,630	1,480	1,500	1,270	1,250	0,740	0,690	0,119
Differe	**0.8**	**1.5**	**1.34**	**1.08**	**0.99**	**0.72**	**0.54**	**1.48**	**0.83**	**0.57**	**0**	**0.18**

nce	**6**	**9**										
Ladder (ng/uL)	**100.**	**50.0**	**25.0**	**12.5**	**6.3**	**3.1**	**100.0**	**50.0**	**25.0**	**12.5**	**6.3**	**3.1**
	Heat Treatment						**Cell Lysis**					
+	0,95	1,46	1,25	1,10	1,39	1,15	1,09	1,11	1,06	0,36	0,17	0,242
-	0,76	0,92	1,17	1,18	1,16	1,26	1,25	0,88	1,35	0,12	0,23	0,143
	0.19	**0.54**	**0.80**	**0**	**0.23**	**0**	**0**	**0.23**	**0**	**0.24**	**0**	**0.099**
	DNA Capture- 3mcg toxin added						**Negative controls (no antigen)**					
+	0,93	0,50	0,93	1,10	1,35	1,07	1,09	1,28	1,01	1,05	0,34	0,799
-	0,76	0,37	0,78	1,01	0,95	0,91	0,90	1,02	0,85	0,27	0,28	0,645

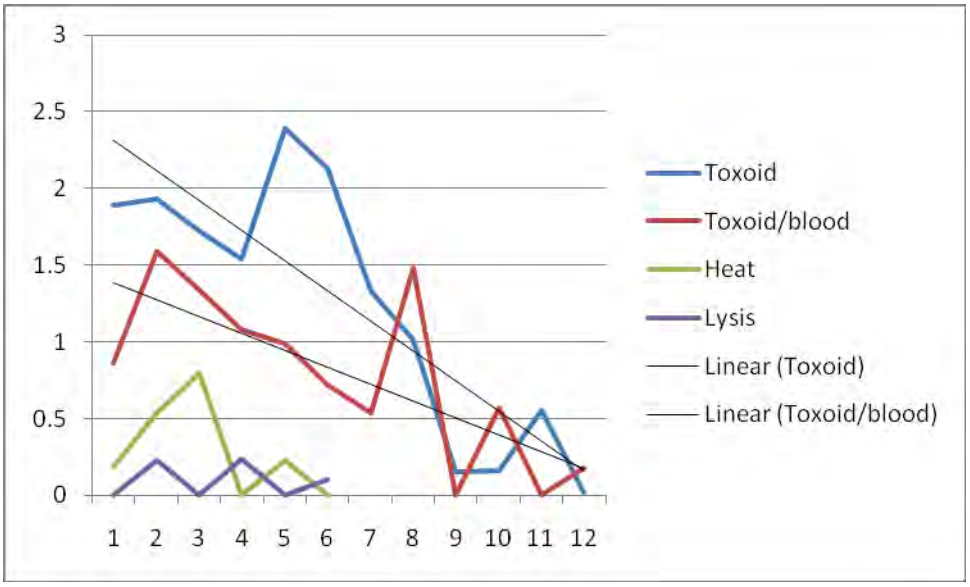

Figure 3. Reduced amount of recombinant ricin toxoid at various stages in DNA extraction as determined by ELISA. **Samples 1 to 12**: doubling dilutions of toxoid added from 100 to 0.1 mcg . **Toxoid:** standard curve of toxoid alone, with estimated linear best fit. **Toxoid/blood**: standard curve of toxoid in blood with estimated best fit. **Heat**: O/N incubation at 56°C. **Lysis**: incubation at 56°C in lysis buffer with proteinase K. OD values plotted have been adjusted for background reactivity in the absence of capture antibody.

For the third toxin, SEB, the presence of toxin in samples was detected by visualization of the toxin by Coomassie blue staining after electrophoresis in a polyacrylamide gel as larger amounts of this protein were added to samples - 1 and 10 mcg. The LD_{50} for this toxin is 0.02 mcg/kg when aerosolized but much higher for oral ingestion, >1 mg/kg (Rusnak et al. 2004). We showed that as little as 0.1 mcg of toxin was detectable by this method (Figure 4), but as much as 10 ug of toxin seeded into blood was degraded to an extent not detected by SDS-PAGE (below 0.1 mcg) after incubation of the sample in cell lysis buffer at 56°C.

Conclusions:

The results of these experiments show that SEB and the ricin toxoid were efficiently degraded by the DNA extraction process. When doses as high as 10 mcg and 100 mcg respectively were added, residual toxin/toxoid was below doses that would affect technical personnel. For

BoNT/A, the ELISA did not specifically detect the toxin in blood samples, nor was the assay very sensitive. While no toxin was detected in samples after incubation in lysis buffer, or after complete DNA isolation, the experiments will need to be repeated to confirm that the amount of BoNT/A in these samples was below toxic doses.

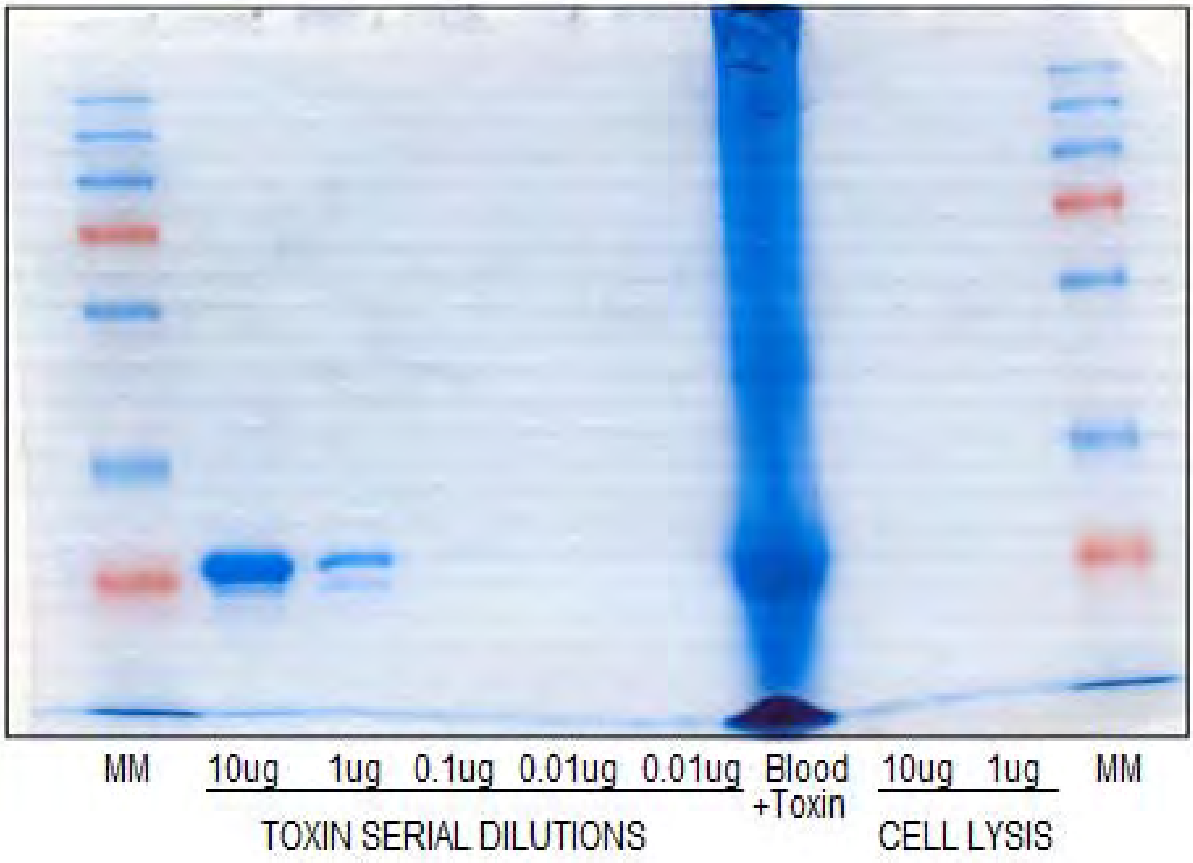

FIGURE 4. Elimination of the Staphylococcal Enterotoxin B (SEB) after incubation in lysis buffer as detected by PAGE. Samples were subjected to electrophoresis through a 12.5% SDS-PAGE gel, and represent one of 3 experiments. MM: Molecular weight markers; Toxin dilutions in PBS; Toxin plus blood = 10 ug in 10 uL of blood, before and after incubation at 56°C in cell lysis buffer.

Objective 3: **Comparison of the quantity and quality of extracted DNA in the absence and presence of contaminating material using two silica-coated magnetic bead extraction methods.** The two methods were an automated robotic system (Qiagen EZRobot EZ1® Advance)

and a manual extraction of DNA using an existing RCMP protocol with the DNA IQ™ kit (Promega). Before completing these experiments, we decided to test whether a lysis buffer in use by the RCMP for the first step in DNA extraction could also be used with the automated system. The experiment consisted of comparing the quantity and quality of DNA extracted from samples with the automated system using the provided buffer (G2, unknown formulation) or the RCMP buffer (100mN NaCL, 10mM Tris HCL (pH 8.0), 10mM EDTA, 0.5% sarkosyl and 40mM DTT). In both cases, proteinase K was added to produce 10mg/mL just prior to use.

Duplicate samples of varying volumes of blood (0.01µL to 50µL) or saliva obtained from a single donor were divided and incubated in the each buffer, then extracted using the robotic system as described in Methods and Materials. DNA was quantified and short tandem repeat (STR) analysis was completed to determine if isolated DNA is of adequate quality be used for identification purposes. The experiment was completed twice. The results in Table 5 indicate that in all samples except the saliva, the amount of DNA was higher in the samples extracted with the RCMP buffer than with the G2 buffer, and the number of samples yielding complete STR profiles (DNA integrity), was also higher with the RCMP buffer, except at the lowest volume of blood. Differences in DNA yield were not statistically significant.

Table 5. DNA quantity and quality after extraction using two lysis buffers (G2 and RCMP) with the BioRobot EZ1® Advance system. Values represent the mean ± SD of duplicate samples in each of 2 experiments (n=4). DNA integrity is the number of samples providing complete STR profiles.

Sample	Buffer	Average DNA quantity (ng)	DNA integrity	Buffer	Average DNA quantity (ng)	DNA integrity
Blood (50µL)	G2	218.1 ± 126.9	4/4	RCMP	488.9 ± 77.1	4/4
Blood (10µL)	G2	53.7 ± 11.4	4/4	RCMP	90.4 ± 10.8	4/4
Blood (1µL)	G2	4.18 ± 1.50	3/4	RCMP	7.38 ± 4.63	4/4
Blood (0.1µL)	G2	0.65 ± 0.18	3/4	RCMP	1.75 ± 0.23	4/4
Blood (0.01µL)	G2	0.20 ± 0.10	2/4	RCMP	0.25 ± 0.08	2/4
Saliva (unknown)	G2	257.9 ± 108.0	4/4	RCMP	186.8 ± 108.9	4/4

On the basis of these preliminary results, the RCMP lysis buffer was used for all further experiments. It was also noted during these preliminary experiments that the 50µL blood volume was too concentrated and required significant dilution to achieve an appropriate DNA concentration for analysis. The volume of 0.01µL yielded inconsistent results. For this reason, the volumes of blood used in all further experiments were 0.1µL, 0.5µL and 1µL.

The next series of experiments were designed to compare the manual to the automated extraction process in non-contaminated samples and samples contaminated with bacteria, spores or toxins. At the same time, the effect of the presence of microbes or toxins on DNA extraction could also be compared. For each experiment, duplicate blood samples from each of two donors were diluted to produce samples of 1µl (10µL of 1:10 blood dilution), 0.5µL (10µL of 1:20 blood dilution) and 0.1µL (10µL of 1:100 blood dilution). Saliva samples were collected from only one donor, so 4 replicates of a single dilution were generated. As before, samples were placed on cotton swabs, and air dried for one hour. In parallel, contaminated samples were established with the addition of 10µL of the selected biological agent to samples after their absorption onto cotton swabs. Blank swabs with no human or bacterial sample, and swabs with the selected biological agent and no human sample were included as controls. After drying, samples were all incubated in the RCMP lysis buffer overnight at 56^{o}C, then sets were extracted by each method. Eluted DNA was filtered, and DNA from samples seeded with bacteria or spores were tested for the absence of infectious agents at the end of the process. Experiments were repeated either two or three times, to give a total 8 or 12 samples. Seven sets of experiments were conducted, with the addition of *Y. pseudotuberculosis*, *P. aeruginosa*, spores from two strains of *B. anthracis*, SEB, BoNT/A and the ricin toxoid.

We first compared the results of DNA extraction from uncontaminated blood and saliva using the automated or the manual process. In the first three experiments, shown in Table 6, the automated system provided statistically significant higher DNA yields, and a higher number of STR profiles could be completed. However, the success rate for complete analysis was not as high as expected with either method. For the next three experiments, shown in Table 7, the yields of DNA isolated using the manual method improved, and equalled those obtained with the automated system. So, these results indicate that the manual process is as efficient as the automated system, but does require training and practice.

Table 6. Initial evaluation of the manual versus automated extraction by comparing the quantity and quality of human DNA isolated from blood and saliva samples.

	Manual extraction		Automated extraction	
Samples	Average DNA quantity (ng)	Completed STR profiles	Average DNA quantity (ng)	Completed STR profiles
Experiment one				
Blood (1µL)	0.365 ± 0.252 0.844 ± 0.328	0/8 (4p)	1.726 ± 0.614 2.688 ± 1.060	7/8 (1p)
Blood (0.5µL)	0.073 ± 0.114 1.317 ± 0.997	0/8 (2p)	0.000 ± 0.000 0.582 ± 0.093	4/8 (1p)
Blood (0.1µL)	0.092 ± 0.108 1.503 ± 1.496	1/8 (2p)	0.633 ± 0.955 0.268 ± 0.192	6/8 (2p)
Saliva (unknown)	8.655 ± 6.456 11.46 ± 2.285	3/8 (2p)	44.95 ± 6.556 155.3 ± 52.58	4/8 (4p)
Experiment two				
Blood (1µL)	0.191 ± 0.197 0.415 ± 0.159 0.296 ± 0.360	0/12	5.089 ± 4.776 4.931 ± 1.626 3.236 ± 1.425	10/12 (1p)
Blood (0.5µL)	0.103 ± 0.063 0.204 ± 0.183 0.273 ± 0.140	0/12	1.521 ± 1.106 2.778 ± 1.556 1.451 ± 0.607	7/12 (1p)
Blood (0.1µL)	0.053 ± 0.039 0.192 ± 0.098 0.096 ± 0.111	0/12	0.149 ± 0.154 0.385 ± 0.135 0.246 ± 0.167	0/12
Saliva (unknown)	2.394 ± 1.502 0.909 ± 0.506 1.681 ± 0.687	7/12	68.43 ± 39.06 71.50 ± 15.12 67.65 ± 23.28	12/12
Experiment three				
Blood (1µL)	91.03 ± 81.39 1.575 ± 0.829 25.25 ± 12.84	2/12 (1p)	9.575 ± 3.632 4.800 ± 0.616 8.050 ± 2.525	5/12 (2p)
Blood (0.5µL)	13.17 ± 18.93 0.605 ± 0.420 5.050 ± 3.436	1/12 (1p)	4.625 ± 0.854 3.325 ± 1.132 0.940 ± 1.310	6/12 (2p)
Blood (0.1µL)	10.52 ± 15.79 1.455 ± 2.430 2.175 ± 2.334	3/12 (1p)	1.675 ± 0.359 0.898 ± 0.482 0.345 ± 0.222	4/12 (1p)
Saliva (unknown)	83.75 ± 73.84 47.00 ± 33.06 616.8 ± 618.8	6/12 (1p)	732.5 ± 293.1 597.5 ± 288.1 1173 ± 130.5	8/12

Numbers are the results of 2 or 3 independent experiments, each done with duplicates of two donors (n=4). Numbers for DNA integrity represents the completed STR profiles of extracted

samples. (p) Partial profile. Values in red are below the quantity of DNA set as the threshold to proceed to analysis.

Table 7: Further evaluation of the manual versus automated extraction by comparing the quantity and quality of human DNA isolated from blood and saliva samples.

	Manual extraction		**Automated extraction**	
Samples	Average DNA quantity (ng)	Completed STR profiles	Average DNA quantity (ng)	Completed STR profiles
Experiment four				
Blood (1µL)	0.211 ± 0.115 1.150 ± 0.296 0.310 ± 0.188	8/12	1.643 ± 1.030 1.230 ± 0.350 1.950 ± 0.854	4/12 (2p)
Blood (0.5µL)	0.270 ± 0.091 1.625 ± 0.350 0.220 ± 0.146	5/12	1.238 ± 1.328 0.540 ± 0.128 1.110 ± 0.229	8/12 (1p)
Blood (0.1µL)	0.150 ± 0.236 0.144 ± 0.061 0.348 ± 0.212	6/12 (1p)	0.193 ± 0.030 0.223 ± 0.086 0.169 ± 0.110	7/12 (2p)
Saliva (unknown)	2.675 ± 1.132 114.3 ± 39.31 19.00 ± 4.690	9/12	107.8 ± 75.94 88.50 ± 37.05 312.5 ± 55.00	12/12
Expeirment five				
Blood (1µL)	0.556 ± 0.271 1.514 ± 2.132 2.320 ± 4.515	11/12	2.325 ± 0.597 2.423 ± 0.537 4.908 ± 1.435	11/12 (1p)
Blood (0.5µL)	0.705 ± 1.014 0.361 ± 0.346 0.427 ± 0.316	8/12 (1p)	1.210 ± 0.196 0.956 ± 0.398 46.44 ± 89.045	5/12
Blood (0.1µL)	1.433 ± 1.788 0.291 ± 0.249 0.099 ± 0.117	7/12 (2p)	0.170 ± 0.087 0.262 ± 0.219 0.625 ± 0.253	0/12 (1p)
Saliva (unknown)	10.54 ± 3.623 23.40 ± 9.156 11.96 ± 8.648	12/12	176.6 ± 71.85 323.3 ± 121.6 217.1 ± 147.1	12/12
Experiment six				
Blood (1µL)	1.180 ± 0.787 0.715 ± 0.452 4.820 ± 8.790	10/12 (2p)	1.675 ±0.320 1.025 ±0.481 1.303 ± 0.467	9/12 (2p)
Blood (0.5µL)	0.895 ± 0.822 0.503 ± 0.401 0.275 ± 0.091	8/12	0.958 ± 0.169 0.983 ± 0.021 0.825 ± 0.304	3/12 (2p)
Blood (0.1µL)	0.285 ± 0.054 0.383 ± 0.126 0.334 ± 0.166	7/12	0.313 ± 0.171 0.508 ± 0.470 0.425 ± 0.373	1/12 (2p)
Saliva (unknown)	166.0 ± 87.47 161.8 ± 69.42 139.5 ± 101.2	12/12	625.0 ± 114.7 337.5 ± 95.35 510.0 ± 115.8	12/12

Numbers are the results of 3 independent experiments, each done with duplicates of two donors (n=4). Numbers for DNA integrity represents the completed STR profiles of extracted samples.

(p) Partial profile. Values in red are below the quantity of DNA set as the threshold to proceed to analysis.

The above results were taken from 6 of the 7 sets of experiments where DNA extraction was compared in the absence and presence of contaminating bioweapon agents. The complete data from these experiments are presented in Tables 8 to 14.

The first four experiments examined the efficiency of human DNA extraction in the presence of *P. aeruginosa*, spores from two strains of *B. anthracis*, and *Y. pseudotuberculosis*. In all cases, as with the uncontaminated samples, DNA was reduced in both quantity and quality when isolated using the manual process compared to the automated process, but again, these are the earliest experiments completed.

The presence of *P. aeruginosa* in samples did compromise the human DNA, and in the case of the 0.1 uL blood samples, the amount of DNA isolated was below the threshold of 0.25 mcg of DNA normally needed to proceed with analysis. The reduction in yield was statistically significant for the 1 and 0.1 ul blood samples and for saliva extracted by the automated method. The integrity of DNA isolated from contaminated samples with either method was also compromised, and in the 1.0 and 0.5 uL blood samples, STR analysis was less successful compared to the uncontaminated samples, even when adequate human DNA was present. The addition of *Y. pseudotuberculosis* into samples had less of an effect, but there was still a trend to lower yields of DNA with statistically less DNA isolated in the automated system from the 1 and 0.1 ul contaminated blood samples compared to uncontaminated samples. The number of successful STR profiles was reduced in contaminated samples compared to uncontaminated samples regardless of the extraction method. In two experiments, the addition of *B. anthracis* spores to samples had little effect on either the quantity or quality of the isolated DNA (Tables 10 and 11).

Table 8. Quantity and quality of human DNA isolated by two methods from samples contaminated with 10^9 CFU of *P. aeruginosa*.

	Manual extraction		Automated extraction	
Samples	Average DNA quantity (ng)	Completed STR profiles	Average DNA quantity (ng)	Completed STR profiles
Uncontaminated Samples				
Blood (1µL)	0.191 ± 0.197 0.415 ± 0.159 0.296 ± 0.360	0/12	5.089 ± 4.776 4.931 ± 1.626 3.236 ± 1.425	10/12 (1p)
Blood (0.5µL)	0.103 ± 0.063 0.204 ± 0.183 0.273 ± 0.140	0/12	1.521 ± 1.106 2.778 ± 1.556 1.451 ± 0.607	7/12 (1p)
Blood (0.1µL)	0.053 ± 0.039 0.192 ± 0.098 0.096 ± 0.111	0/12	0.149 ± 0.154 0.385 ± 0.135 0.246 ± 0.167	0/12
Saliva (unknown)	2.394 ± 1.502 0.909 ± 0.506 1.681 ± 0.687	7/12	68.43 ± 39.06 71.50 ± 15.12 67.65 ± 23.28	12/12
***P. aeruginosa* Contaminated Samples**				
Blood (1µL)	0.056 ± 0.092 0.313 ± 0.231 0.165 ± 0.190	0/12	1.521 ± 1.106 1.579 ± 0.703 0.168 ± 0.155	1/12
Blood (0.5µL)	0.219 ± 0.254 0.103 ± 0.153 0.058 ± 0.066	0/12	1.263 ± 1.887 0.959 ± 0.205 0.367 ± 0.492	1/12
Blood (0.1µL)	0.011 ± 0.004 0.104 ±0.084 0.093 ± 0.168	0/12	0.079 ± 0.096 0.312 ± 0.197 0.127 ± 0.108	0/12
Saliva (unknown)	0.579 ± 0.457 0.909 ± 0.506 0.537 ± 0.267	1/12	14.57 ± 17.20 28.14 ± 6.788 17.50 ± 12.73	9/12
Negative Controls				
Blank (no DNA)	0.000 ± 0.000	1/3 Cont.	0.000 ± 0.000	0/3

Numbers represent the means ± SD of duplicate samples from two donors (n=4) of blood, or quadruplicates of saliva from a single donor, repeated in 3 experiments. DNA integrity is the number of completed STR profiles out of the total number of extracted samples. (p) Partial profile. Values in red are below the quantity of DNA set as the threshold to proceed to analysis. (p) Partial profile.

Table 9. Quantity and quality of human DNA isolated by two methods from samples contaminated with 10^9 CFU of *Y. Pseudotuberculosis.*

	Manual extraction		Automated extraction	
Samples	Average DNA quantity (ng)	Completed STR profiles	Average DNA quantity (ng)	Completed STR profiles
Uncontaminated Samples				
Blood (1µL)	91.03 ± 81.39 1.575 ± 0.829 25.25 ± 12.84	2/12 (1p)	9.575 ± 3.632 4.800 ± 0.616 8.050 ± 2.525	5/12 (2p)
Blood (0.5µL)	13.17 ± 18.93 0.605 ± 0.420 5.050 ± 3.436	1/12 (1p)	4.625 ± 0.854 3.325 ± 1.132 0.940 ± 1.310	6/12 (2p)
Blood (0.1µL)	10.52 ± 15.79 1.455 ± 2.430 2.175 ± 2.334	3/12 (1p)	1.675 ± 0.359 0.898 ± 0.482 0.345 ± 0.222	4/12 (1p)
Saliva (unknown)	83.75 ± 73.84 47.00 ± 33.06 616.8 ± 618.8	6/12 (1p)	732.5 ± 293.1 597.5 ± 288.1 1173 ± 130.5	8/12
Contaminated Samples				
Blood (1µL)	187.4 ± 212.3 2.950 ± 2.596 2.325 ± 2.995	2/12	33.00 ± 2.708 33.25 ± 15.52 18.250 ± 2.062	10/12
Blood (0.5µL)	27.85 ± 32.50 2.110 ± 3.407 2.144 ± 3.393	6/12	19,00 ± 4.320 10.38 ± 5.915 1.038 ± 0.838	8/12
Blood (0.1µL)	1.815 ± 2.060 2.114 ± 3.209 2.299 ± 2.852	5/12 (2p)	5.100 ± 2.865 5.975 ± 5.456 6.725 ± 9.519	2/12 (1p)
Saliva (unknown)	117.0 ± 112.3 474.3 ± 483.0 289.0 ± 295.6	8/12	635.0 ± 103.4 1150 ± 493.3 877.5 ± 578.3	8/12 (1p)
Negative Controls				
Blank (no DNA)	0.106 ± 0.122	0/3	0.082 ± 0.080	0/3

Numbers represent the means ± SD of duplicate samples from two donors (n=4) of blood, or quadruplicates of saliva from a single donor, repeated in 3 experiments. DNA integrity is the number of completed STR profiles out of the total number of extracted samples. (p) Partial profile. Values in red are below the quantity of DNA set as the threshold to proceed to analysis.

Table 10. Quantity and quality of human DNA isolated by two methods from samples contaminated with 10^9 spores from *B. anthracis* Stern strain.

	Manual extraction		Automated extraction	
Samples	Average DNA quantity (ng)	Completed STR profiles	Average DNA quantity (ng)	Completed STR profiles
Uncontaminated Samples				
Blood (1µL)	0.365 ± 0.252 0.844 ± 0.328	0/8 (4p)	1.726 ± 0.614 2.688 ± 1.060	7/8 (1p)
Blood (0.5µL)	0.073 ± 0.114 1.317 ± 0.997	0/8 (2p)	0.000 ± 0.000 0.582 ± 0.093	4/8 (1p)
Blood (0.1µL)	0.092 ± 0.108 1.503 ± 1.496	1/8 (2p)	0.633 ± 0.955 0.268 ± 0.192	6/8 (2p)
Saliva (unknown)	8.655 ± 6.456 11.46 ± 2.285	3/8 (2p)	44.95 ± 6.556 155.3 ± 52.58	4/8 (4p)
Contaminated Samples-*B. anthracis* Sterne spores				
Blood (1µL)	0.088 ± 0.107 0.380 ± 0.111	2/8 (1p)	1.077 ± 0.477 2.376 ±1.057	7/8 (1p)
Blood (0.5µL)	0.145 ± 0.098 0.616 ± 0.340	2/8	0.134 ± 0.268 0.484 ± 0.409	6/8
Blood (0.1µL)	0.062 ± 0.086 0.745 ± 0.237	0/8 (1p)	0.065 ± 0.131 0.000 ± 0.000	4/8 (4p)
Saliva (unknown)	4.165 ± 1.358 8.046 ± 4.635	1/8 (1p)	54.44 ± 35.55 107.6 ± 25.67	6/8 (2p)
Negative Controls				
Blank (no DNA)	0,000 ± 0,000	0/2	0,000 ± 0,000	0/2

Numbers represent the means ± SD of duplicate samples from two donors (n=4) of blood, or quadruplicates of saliva from a single donor, repeated in 2 experiments. DNA integrity is the number of completed STR profiles out of the total number of extracted samples. (p) Partial profile. Values in red are below the quantity of DNA set as the threshold to proceed to analysis.

Table 11. Quantity and quality of human DNA isolated by two methods from samples

	Manual extraction		Automated extraction	
Samples	Average DNA quantity (ng)	Completed STR profiles	Average DNA quantity (ng)	Completed STR profiles
Uncontaminated Samples				
Blood (1µL)	1.638 ± 0.737 0.441 ± 0.511 27.76 ± 54.83	11/12 (1p)	5.265 ± 1.804 3.845 ± 2.403 4.813 ± 3.349	12/12
Blood (0.5µL)	0.624 ± 0.273 0.000 ± 0.000 0.177 ± 0.160	7/12 (5p)	1.204 ± 0.188 0.751 ± 0.272 1.190 ± 0.476	11/12 (1p)
Blood (0.1µL)	0.422 ± 0.281 0.174 ± 0.243 42.89 ± 85.74	6/12 (5p)	0.459 ± 0.542 0.258 ± 0.313 0.436 ± 0.424	12/12
Saliva (unknown)	27.90 ± 13.36 8.824 ± 2.734 22.78 ± 28.47	12/12	273.9 ± 44.96 187.8 ± 63.11 213.4 ± 134.2	12/12
Contaminated Samples- *B. anthracis*				
Blood (1µL)	3.826 ± 3.188 0.165 ± 0.238 0.881 ± 0.594	11/12 (1p)	2.348 ± 0.320 5.311 ± 1.632 6.564 ± 2.943	12/12
Blood (0.5µL)	2.730 ± 4.099 0.108 ± 0.134 0.170 ± 0.188	8/12 (4p)	0.378 ± 0.165 0.064 ± 0.228 1.573 ± 0.807	12/12
Blood (0.1µL)	0.982 ± 0.608 0.000 ± 0.000 0.208 ± 0.168	1/12 (6p)	0.480 ± 0.347 0.487 ± 0.324 0.736 ± 0.360	11/12
Saliva (unknown)	18.36 ± 12.29 11.47 ± 5.727 20.94 ± 18.74	11/12 (1p)	176.1 ± 13.87 190.8 ± 38.12 220.6 ± 168.2	12/12
Negative Controls				
Blank (no DNA)	0.240 ± 0.210	0/3	0.152 ± 0.122	0/3

contaminated with 10^9 spores from *B. anthracis* NML 03-0083.

Numbers represent the means ± SD of duplicate samples from two donors (n=4) of blood, or quadruplicates of saliva from a single donor, repeated in 3 experiments. DNA integrity is the number of completed STR profiles out of the total number of extracted samples. (p) Partial profile. Values in red are below the quantity of DNA set as the threshold to proceed to analysis.

The final experiments to address this third objective compared the DNA isolated from samples contaminated with the BoNT/A, SEB or the ricin toxoid. Samples were established as previously, but with the addition of a single amount of toxin/toxoid to generate the contaminated set, as follows. For BoTN/A and SEB, 1mcg was added per sample; for the ricin toxoid, 3 mcg was added per sample. As seen in Tables 12-14, the manual extraction method was overall as efficient as the automated in these experiments, whether samples were contaminated or not, and the presence of the toxins or toxoid had no effects on the quantity and quality of isolated DNA.

Table 12. Quantity and quality of human DNA isolated by two methods from samples contaminated with 1 mcg of Botulinum Toxin A.

	Manual extraction		Automated extraction	
Samples	Average DNA quantity (ng)	Completed STR profiles	Average DNA quantity (ng)	Completed STR profiles
Uncontaminated Samples				
Blood (1µL)	0.211 ± 0.115 1.150 ± 0.296 0.310 ± 0.188	8/12	1.643 ± 1.030 1.230 ± 0.350 1.950 ± 0.854	4/12 (2p)
Blood (0.5µL)	0.270 ± 0.091 1.625 ± 0.350 0.220 ± 0.146	5/12	1.238 ± 1.328 0.540 ± 0.128 1.110 ± 0.229	8/12 (1p)
Blood (0.1µL)	0.150 ± 0.236 0.144 ± 0.061 0.348 ± 0.212	6/12 (1p)	0.193 ± 0.030 0.223 ± 0.086 0.169 ± 0.110	7/12 (2p)
Saliva (unknown)	2.675 ± 1.132 114.3 ± 39.31 19.00 ± 4.690	9/12	107.8 ± 75.94 88.50 ± 37.05 312.5 ± 55.00	12/12
Contaminated Samples				
Blood (1µL)	0.223 ± 0.059 1.983 ± 0.916 1.025 ± 0.577	6/12 (1p)	2.650 ± 0.777 1.985 ± 0.960 2.100 ± 0.483	8/12 (1p)
Blood (0.5µL)	0.177 ± 0.243 1.200 ± 0.216 0.288 ± 0.145	9/12	1.190 ± 0.612 0.613 ± 0.038 1.450 ± 0.580	7/12 (1p)
Blood (0.1µL)	0.638 ± 1.175 1.068 ± 0.944 0.086 ± 0.073	3/12 (1p)	0.122 ± 0.080 0.121 ± 0.097 0.182 ± 0.132	4/12 (1p)
Saliva (unknown)	7.550 ± 3.379 167.5 ± 38.62 29.40 ± 25.87	8/12 (2p)	217.5 ± 35.94 150.0 ± 31.62 100.8 ± 34.19	11/12 (1p)
Negative Controls				
Blank (no DNA)	0.000 ± 0.000	0/3	0.000 ± 0.000	0/3

Numbers represent the means ± SD of duplicate samples from two donors (n=4) of blood, or quadruplicates of saliva from a single donor, repeated in 3 experiments. DNA integrity is the number of completed STR profiles out of the total number of extracted samples. (p) Partial profile. Values in red are below the quantity of DNA set as the threshold to proceed to analysis.

Table 13: Quantity and quality of human DNA isolated by two methods from uncontaminated samples and contaminated samples with SEB (1mcg/sample).

	Manual extraction		Automated extraction	
Samples	Average DNA quantity (ng)	Completed STR profiles	Average DNA quantity (ng)	Completed STR profiles
Uncontaminated Samples				
Blood (1µL)	0.556 ± 0.271 1.514 ± 2.132 2.320 ± 4.515	11/12	2.325 ± 0.597 2.423 ± 0.537 4.908 ± 1.435	11/12 (1p)
Blood (0.5µL)	0.705 ± 1.014 0.361 ± 0.346 0.427 ± 0.316	8/12 (1p)	1.210 ± 0.196 0.956 ± 0.398 46.44 ± 89.045	5/12
Blood (0.1µL)	1.433 ± 1.788 0.291 ± 0.249 0.099 ± 0.117	7/12 (2p)	0.170 ± 0.087 0.262 ± 0.219 0.625 ± 0.253	0/12 (1p)
Saliva (unknown)	10.54 ± 3.623 23.40 ± 9.156 11.96 ± 8.648	12/12	176.6 ± 71.85 323.3 ± 121.6 217.1 ± 147.1	12/12
Contaminated Samples				
Blood (1µL)	0.393 ± 0.260 0.396 ± 0.404 1.554 ± 2.418	10/12 (1p)	2.588 ± 0.312 2.458 ± 0.727 3.770 ± 2.106	11/12
Blood (0.5µL)	0.112 ± 0.093 1.219 ± 2.101 0.499 ± 0.265	10/12	1.350 ± 0.185 0.905 ± 0.543 1.147 ± 0.152	5/12
Blood (0.1µL)	0.064 ± 0.099 0.314 ± 0.135 0.453 ± 0.905	9/12 (1p)	0.092 ± 0.054 0.190 ± 0.072 0.254 ± 0.133	3/12
Saliva (unknown)	5.153 ± 4.572 6.975 ± 4.141 8.930 ± 2.946	8/12 (2p)	167.6 ± 198.6 205.3 ± 107.0 137.4 ± 137.4	12/12
Negative Controls				
Blank (no DNA)	0.067 ± 0.115	0/3	0.033 ± 0.029	0/3

Numbers represent the means ± SD of duplicate samples from two donors (n=4) of blood, or quadruplicates of saliva from a single donor, repeated in 3 experiments. DNA integrity is the number of completed STR profiles out of the total number of extracted samples. (p) Partial profile. Values in red are below the quantity of DNA set as the threshold to proceed to analysis.

Table 14. Quantity and quality of human DNA isolated by two methods from uncontaminated samples and samples contaminated ricin toxoid, 3 mcg/sample.

	Manual extraction		Automated extraction	
Samples	Average DNA quantity (ng)	Completed STR profiles	Average DNA quantity (ng)	Completed STR profiles
Uncontaminated Samples				
Blood (1µL)	1.180 ± 0.787 0.715 ± 0.452 4.820 ± 8.790	10/12 (2p)	1.675 ±0.320 1.025 ±0.481 1.303 ± 0.467	9/12 (2p)
Blood (0.5µL)	0.895 ± 0.822 0.503 ± 0.401 0.275 ± 0.091	8/12	0.958 ± 0.169 0.983 ± 0.021 0.825 ± 0.304	3/12 (2p)
Blood (0.1µL)	0.285 ± 0.054 0.383 ± 0.126 0.334 ± 0.166	7/12	0.313 ± 0.171 0.508 ± 0.470 0.425 ± 0.373	1/12 (2p)
Saliva (unknown)	166.0 ± 87.47 161.8 ± 69.42 139.5 ± 101.2	12/12	625.0 ± 114.7 337.5 ± 95.35 510.0 ± 115.8	12/12
Contaminated Samples				
Blood (1µL)	2.575 ± 1.457 0.708 ± 0.614 0.385 ± 0.119	10/12	1.775 ± 0.672 1.448 ± 0.425 1.680 ± 0.864	8/12
Blood (0.5µL)	0.803 ± 0.594 0.480 ± 0.482 0.267 ± 0.140	5/12 (1p)	0.993 ± 0.671 0.970 ± 0.214 0.424 ± 0.406	4/12 (3p)
Blood (0.1µL)	0.488 ± 0.438 0.193 ± 0.015 0.191 ± 0.154	4/12 (2p)	0.538 ± 0.128 0.445 ± 0.265 0.283 ± 0.105	3/12
Saliva (unknown)	555.0 ± 326.6 119.8 ± 36.06 79.75 ± 49.94	12/12	592.5 ± 145.7 455.0 ± 86.60 575.0 ± 136.3	12/12
Negative Controls				
Blank (no DNA)	0.071 ± 0.000	0/3	0.026 ± 0.023	0/3

Numbers represent the means ± SD of duplicate samples from two donors (n=4) of blood, or quadruplicates of saliva from a single donor, repeated in 3 experiments. DNA integrity is the number of completed STR profiles out of the total number of extracted samples. (p) Partial profile. Values in red are below the quantity of DNA set as the threshold to proceed to analysis.

Conclusions:

Comparison of a manual and an automated method for extraction of human DNA over the course of several months demonstrated no difference in the two methods with regard to quality or quantity of DNA isolated. However, the manual method was less efficient until the individual completing the experiments had become more practiced. The presence of spores or toxins in the samples had no effect on the ability to isolate DNA using either method. The presence of *P. aeruginosa* and *Y. pseudotuberculosis* did result in reduced quantities of DNA, and a reduced efficiency of STR analysis. In no case was infectious material detected in isolated DNA, confirming that the DNA extraction method eliminates infectivity of spores and bacteria.

Objective 4: To determine if prolonged exposure of biological samples to live bacteria or toxins will affect DNA yield. The presence of bacteria, even for a short period of time, seemed to affect the ability to extract usable DNA from samples. Hence the final objective was to determine if prolonged exposure of biological samples to live bacteria, bacterial spores or toxins would affect the DNA yield or quality, using the automated procedure. A control experiment was completed to determine if incubation of blood or saliva at room temperature for one week would affect DNA extraction. Table 15 shows that there were no observable effects on the DNA yields and integrity after such an incubation.

To determine the effect of longer periods of exposure to bacteria, spores or toxins on DNA in samples, blood from two donors and saliva from one donor were prepared as before. 10μL of selected biological agent was added to each sample. Contaminated samples and controls were placed in sterile tubes and were air-dried and incubated at room temperature. At the appropriate time, day 1, 3 or 7, DNA was extracted and analyzed for quantity and quality. Analysis was done for samples contaminated with *Y. pseudotuberculosis, B. anthracis* spores, BoTN/A, SEB and ricin toxoid.

The previous experiments had indicated that the presence of *Y. pseudotuberculosis* in blood and saliva reduced the yields of human DNA. In this experiment, Table 16, there was a reduction in the amount of DNA isolated from samples incubated for 3 days and 7 days with the bacteria, compared to samples incubated for 1 day, but the amount of DNA was still above the threshold required to proceed with analysis; the number of STR profiles was as good from DNA isolated after 7 days as for DNA isolated at 1 day. The incubation of blood and saliva with spores,

BoNT/A, SEB or the ricin toxoid for 3 and 7 days had no effect on the quantity or quality of DNA isolated compared to a shorter incubation of 1 day (Tables 17 to 20).

Conclusions:

Exposure of blood and saliva to bacteria, spores or toxins for up to one week has minimal effects on the efficiency of isolation of DNA from such samples; in all cases the quantity and quality of isolated DNA were adequate for STR analysis.

Table 15: Effects of incubation at room temperature for up to one week on DNA extraction from blood and saliva.

Samples	Average DNA quantity (ng)	Completed STR profiles
DAY 1		
Blood (1µL)	33.58 ± 4.402 4.258 ± 2.030 4.185 ± 1.462	10/12 (2p)
Blood (0.5µL)	11.36 ± 4.550 2.360 ± 0.820 1.960 ± 0.378	7/12 (3p)
Blood (0.1µL)	21.94 ± 41.18 0.626 ± 0.133 1.118 ± 0.839	1/12
Saliva (unknown)	192.5 ± 68.19 262.3 ± 96.04 367.0 ± 65.96	11/12
DAY 3		
Blood (1µL)	12.52 ± 2.543 20.38 ± 8.562 20.73 ± 6.443	12/12
Blood (0.5µL)	8.050 ± 3.909 10.09 ± 4.012 8.560 ± 6.635	11/12
Blood (0.1µL)	1.955 ± 1.023 2.462 ± 1.161 3.395 ± 3.665	7/12 (2p)
Saliva (unknown)	79.73 ± 21.67 498.8 ± 96.15 146.9 ± 99.63	9/12
DAY 7		
Blood (1µL)	23.25 ± 4.535 20.68 ± 4.219 36.78 ± 9.807	12/12
Blood	9.758 ± 2.634	11/12

(0.5µL)	9.563 ± 4.809 12.72 ± 4.692	
Blood (0.1µL)	2.343 ± 0.493 3.355 ± 2.592 8.140 ± 10.91	8/12
Saliva (unknown)	100.0 ± 57.77 239.0 ± 51.75 189.4 ± 143.5	9/12 (2p)
CONTROLS (7 days exposure)		
Biological agents (10^9)	N/A	N/A
Aged blood (1µL)	10.93 ± 2.200	3/3
Negative (Blank)	0.000 ± 0.000	0/3

Numbers are the means + SD of duplicate samples from 2 donors for blood, or quadruplicates of saliva from a single donor. The experiment was completed 3 times using the EZ Robot Advanced system (Qiagen).

Table 16: Effects of prolonged exposure to *Y. pseudotuberculosis* on extraction of human DNA from blood and saliva.

Samples	Average DNA quantity (ng)	Completed STR profiles
DAY 1		
Blood (1µL)	38.75 ± 5.679 42.25 ± 5.560 24.50 ± 6.758	4/12 (7p)
Blood (0.5µL)	25.25 ± 2.217 25.25 ± 5.315 12.75 ± 2.872	2/12 (8p)
Blood (0.1µL)	4.275 ± 1.688 5.200 ±0.469 2.850 ± 0.777	2/12 (2p)
Saliva (unknown)	990.0 ± 290.5 825.0 ± 229.0 440.0 ± 74.83	11/12 (1p)
DAY 3		
Blood (1µL)	8.650 ± 2.301 11.68 ± 6.764 10.025 ± 4.964	11/12 (0p)
Blood (0.5µL)	5.475 ± 3.266 4.225 ± 2.304 5.500 ± 2.317	5/12 (1p)
Blood (0.1µL)	1.140 ± 0.346 1.095 ± 0.381 1.750 ± 0.493	4/12 (1p)
Saliva (unknown)	365.0 ± 151.8 327.5 ± 61.31 363.5 ± 129.2	11/12 (0p)

DAY 7		
Blood (1µL)	18.50 ± 5.802 14.45 ± 17.44 4.515 ± 2.784	11/12 (0p)
Blood (0.5µL)	7.200 ± 4.569 4.450 ± 2.496 4.225 ± 1.926	7/12 (2p)
Blood (0.1µL)	1.388 ± 0.581 1.225 ± 0.310 1.308 ± 0.509	3/12 (0p)
Saliva (unknown)	132.5 ± 23.63 175.0 ± 43.59 146.0 ± 64.48	12/12 (0p)
CONTROLS (7 days exposure)		
Biological agents (10^9)	3.011 ± 2.767	0/3
Aged blood (1µL)	77.00 ± 55.22	3/3
Negative (Blank)	78.37 ± 106.8	0/3

Samples were seeded with 10^9CFU of bacteria. Numbers are the means $\pm$ SD of duplicate samples from two donors for blood or quadruplicates of one donor for saliva (n=4). The experiment was repeated 3 times. Numbers for DNA integrity represent the completed STR profiles of extracted samples. (p) Partial profile

Table 17. Effects of exposure to *B. anthracis* on DNA isolation.

Samples were seeded with 10^9CFU of bacteria. Numbers are the means ± SD of duplicate samples from two donors for blood or quadruplicates of one donor for saliva (n=4). The experiment was repeated 3 times. Numbers for DNA integrity represent the completed STR profiles of extracted samples. (p) Partial profile

Samples	Average DNA quantity (ng)	Completed STR profiles
DAY 1		
Blood (1µL)	0.431 ± 0.530 2.988 ± 3.531 0.312 ± 0.210	10/12
Blood (0.5µL)	0.115 ± 0.139 1.023 ± 1.810 0.083 ± 0.096	2/12
Blood (0.1µL)	0.000 ± 0.000 0.000 ± 0.000 0.094 ± 0.188	0/12
Saliva (unknown)	41.42 ± 30.26 67.76 ± 56.19 17.75 ± 35.50	10/12
DAY 3		
Blood (1µL)	0.611 ± 0.499 0.273 ± 0.234 0.263 ± 0.416	10/12
Blood (0.5µL)	0.306 ± 0.357 0.087 ± 0.102 0.158 ± 0.315	3/12
Blood (0.1µL)	0.269 ± 0.407 0.071 ± 0.085 0.000 ± 0.000	0/12
Saliva (unknown)	15.91 ± 18.17 18.73 ± 14.50 75.76 ± 39.02	8/12
DAY 7		
Blood (1µL)	1.754 ± 1.538 7.773 ± 9.536 2.820 ± 2.956	12/12
Blood (0.5µL)	0.269 ± 0.432 1.149 ± 1.466 0.676 ± 1.353	0/12
Blood (0.1µL)	0.140 ± 0.280 0.054 ± 0.107 0.333 ± 0.385	0/12
Saliva (unknown)	15.06 ± 11.71 12.58 ± 15.33 12.75 ± 3.951	0/12
CONTROLS (7 days exposure)		
Biological agents (10^9)	0.000 ± 0.000	0/3
Aged blood (1µL)	1.177 ± 1.038	3/3
Negative (Blank)	0.000 ± 0.000	0/3

Table 18: Quantity and quality of human DNA isolated from samples exposed to Botulinum toxin A for variable times.

Samples	Average DNA quantity (ng)	Completed STR profiles
DAY 1		
Blood (1µL)	16.50 ± 4.655 19.50 ± 10.47 13.75 ± 3.403	12/12 (0p)
Blood (0.5µL)	6.750 ± 2.897 8.275 ± 2.597 7.550 ± 1.377	12/12 (0p)
Blood (0.1µL)	2.125 ± 0.741 1.600 ± 0.424 1.550 ± 0.480	6/12 (1p)
Saliva (unknown)	487.5 ± 143.9 247.5 ± 9.574 312.5 ±110.9	11/12 (0p)
DAY 3		
Blood (1µL)	12.08 ± 2.239 17.25 ± 3.594 12.33 ±2.724	10/12 (2p)
Blood (0.5µL)	8.100 ± 2.404 7.600 ± 1.476 5.650 ± 2.439	11/12 (1p)
Blood (0.1µL)	1.625 ± 0.499 1.118 ± 0.335 1.068 ± 0.441	1/12 (3p)
Saliva (unknown)	207.5 ± 122.3 407.5 ± 68.50 196.8 ± 119.0	12/12
DAY 7		
Blood (1µL)	11.80 ± 3.930 15.38 ± 5.121 20.78 ±15.39	12/12 (0p)
Blood (0.5µL)	9.075 ± 3.298 4.575 ± 1.706 6.825 ± 1.072	12/12 (0p)
Blood (0.1µL)	1.280 ± 0.431 1.575 ± 0.427 1.070 ± 0.286	0/12 (6p)
Saliva (unknown)	129.5 ± 95.64 170.3 ± 133.8 255.5 ± 154.2	12/12 (0p)
CONTROLS (7 days exposure)		
Biological agents (10^9)	540.7 ± 917.5	0/3
Aged blood (1µL)	7.967 ± 1.498	3/3
Negative (Blank)	39.53 ± 53.33	0/3

Samples were seeded with 1µg of toxin. Numbers are the means ± SD of duplicate samples from two donors for blood or quadruplicates of one donor for saliva (n=4). The experiment was repeated 3 times Numbers for DNA integrity represent the completed STR profiles of extracted

samples. (p) Partial profile

Table 19: Quantity and quality of human DNA isolated from samples exposed to SEB for variable times.

Samples	Average DNA quantity (ng)	Completed STR profiles
DAY 1		
Blood (1µL)	32.83 ± 19.11 5.480 ± 1.111 3.673 ± 0.109	11/12 (0p)
Blood (0.5µL)	12.70 ± 5.121 4.715 ± 2.274 2.720 ± 1.721	8/12 (1p)
Blood (0.1µL)	1.254 ± 1.058 1.463 ± 0.328 0.987 ± 0.710	0/12 (0p)
Saliva (unknown)	126.1 ± 55.60 343.0 ± 69.59 659.8 ± 92.61	12/12 (0p)
DAY 3		
Blood (1µL)	16.55 ± 2.649 20.75 ± 5.368 10.51 ± 3.882	12/12
Blood (0.5µL)	9.990 ± 1.077 9.063 ± 1.840 26.60 ± 42.75	12/12
Blood (0.1µL)	0.841 ± 0.789 3.065 ± 0.779 2.433 ± 0.989	2/12 (4p)
Saliva (unknown)	71.73 ± 26.83 551.5 ± 67.58 85.48 ± 50.77	12/12
DAY 7		
Blood (1µL)	15.72 ± 16.21 12.59 ± 8.387 7.847 ± 12.15	6/12 (0p)
Blood (0.5µL)	6.758 ± 7.743 7.625 ± 5.781 6.385 ± 7.455	5/12 (2p)
Blood (0.1µL)	0.713 ± 1.425 3.210 ± 0.473 0.510 ± 0.866	0/12 (1p)
Saliva (unknown)	9.942 ± 19.57 301.5 ± 87.98 113.8 ± 224.1	6/12 (1p)
CONTROLS (7 days exposure)		

Biological agents (10^9)	0.000 ± 0.000	0/3
Aged blood (1µL)	6.372 ± 5.618	3/3
Negative (Blank)	0.000 ± 0.000	0/3

Samples were seeded with 1µg of toxin. Numbers are the means ± SD of duplicate samples from two donors for blood or quadruplicates of one donor for saliva (n=4). The experiment was repeated 3 times Numbers for DNA integrity represents the completed STR profiles of extracted samples. (p) Partial profile

Table 20: Quantity and quality of human DNA extracted from samples exposed to ricin toxoid.

Samples	Average DNA quantity (ng)	Completed STR profiles
DAY 1		
Blood (1µL)	18.50 ± 9.469 10.78 ± 2.956 10.38 ± 4.507	12/12 (0p)
Blood (0.5µL)	5.650 ± 1.396 5.775 ± 1.075 3.900 ± 1.254	11/12 (1p)
Blood (0.1µL)	0.678 ± 0.111 0.925 ± 0.492 0.790 ± 0.422	3/12 (2p)
Saliva (unknown)	175.0 ± 34.16 222.5 ± 100.8 152.3 ± 59.44	12/12 (0p)
DAY 3		
Blood (1µL)	11.95 ± 4.001 17.25 ± 8.461 14.33 ± 4.888	12/12
Blood (0.5µL)	5.650 ± 1.646 11.53 ± 3.513 5.425 ± 2.877	11/12 (1p)
Blood (0.1µL)	0.630 ± 0.314 0.988 ± 0.411 0.545 ± 0.197	2/12 (1p)
Saliva (unknown)	197.50 ± 60.21 125.0 ± 19.15 147.5 ± 28.72	11/12 (1p)
DAY 7		
Blood (1µL)	15.25 ± 4.425 20.00 ± 2.309 15.95 ± 5.900	12/12 (0p)
Blood (0.5µL)	7.250 ± 1.949 6.850 ± 1.271 7.050 ± 1.794	12/12 (0p)
Blood (0.1µL)	0.768 ± 0.118 0.763 ± 0.299 0.571 ± 0.495	3/12 (0p)
Saliva (unknown)	156.3 ± 55.58 117.5 ± 110.2 370.0 ± 224.7	11/12 (1p)
CONTROLS (7 days exposure)		

Biological agents (10^9)	17.39 ± 29.12	0/3
Aged blood (1µL)	10.03 ± 2.581	3/3
Negative (Blank)	7.397 ± 12.65	0/3

Samples were seeded with 3mcg of toxoid. Numbers are the means ± SD of duplicate samples from two donors for blood or quadruplicates of one donor for saliva (n=4). The experiment was completed 3 times. Numbers for DNA integrity represent the completed STR profiles of extracted samples. (p) Partial profile

4 Summary and Conclusions:

In over 330 samples seeded with infectious bacteria or spores in 8 experiments, no infectious material was detected in the final DNA samples. These results confirm that the DNA extraction procedure results in samples that are safe for DNA analysis.

Experiments conducted as part of this project showed that incubation of potentially contaminated samples in lysis buffer/Proteinase K at 56^{o}C eliminated as many as 10^{9} infectious bacteria, and greatly reduced the number of spores. However, spores did survive this step, so samples with unknown contamination should be considered to be infectious until after the filtration step.

2) **The DNA extraction process, in particular incubation of samples in lysis buffer/Proteinase K at 56^{o}C, resulted in degradation of SEB and the ricin toxoid to levels below toxic doses.** The ricin toxoid used in these experiments differs from the ricin toxin by only 2 amino acids, so the sensitivity of the toxin is expected to be identical to the toxoid.

The results obtained with BoNT/A showed clear reductions in the amount of toxin after incubation in lysis buffer/proteinase K, and after DNA capture. However, the ELISA assay used to determine the amount of residual toxin present in purified DNA did not display the expected specificity or sensitivity to allow us to conclusively determine the amount residual toxin in the DNA samples.

As procedures to isolate nucleic acids are specifically designed to remove protein and lipid from DNA or RNA, it is expected that all toxins, including BoNT/A, would be denatured or removed from the DNA during extraction to an extent that samples would be safe for handling.

3) **The presence of representative bacteria, bacterial spores or toxins did not compromise the quantity or quality of human DNA isolated from contaminated blood or saliva.** While some experiments indicated that the presence of *P. aeruginosa* or *Y. pseudotuberculosis* in blood and saliva resulted in some loss of human DNA compared to DNA isolated from uncontaminated samples, the amount of DNA was not reduced below the threshold needed to ensure efficient amplification and STR analysis. This was confirmed for *Y. pseudotuberculosis* in an experiment examining exposure to the bacteria for up to one week prior to DNA extraction.

Spores and toxins had no negative effects on human DNA after incubation of up to one week. This confirms the findings of Hause (2007)

4) **The Qiagen EZ Advanced robotic system for automated extraction of DNA from a small number of samples was efficient at isolating human DNA from blood samples ranging in volume from 10.0 to 0.1 uL of blood in the absence and presence of contamination agents**, but did not allow consistent isolation of adequate quantities of DNA from smaller volumes of blood (data not shown).

The RCMP lysis buffer could be substituted in this system for the provided buffer without any detrimental effect on results.

The quantities and quality of DNA isolated using the EZ Advance system were not improved over manual extraction of DNA using the DNA IQ kit (Promega), provided that the person carrying out the manual extraction was somewhat experienced with the method. The automated system may have some advantages in eliminating variability due to human error, and also requires less handling of samples that could still be infectious after the first incubation step.

Issues remaining

There is one class of potential biological weapons not yet examined in this protocol, the viruses. The viruses considered to be of major concern, such as smallpox, Ebola and other hemorrhagic fever viruses, all possess lipid envelopes, which will be readily degraded in the RCMP lysis buffer. There are non-enveloped viruses that could also be utilized by terrorists, such as poliovirus, norovirus and many viruses that infect agricultural crops. These viruses are more resistant to detergents and solvents than viruses with an envelope, but should rupture due to the presence of DTT and proteinase K in the buffer. The final filtration step through a 0.22 micron filter will not eliminate residual viruses contaminating the extracted DNA.

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The purpose of this research project was to further characterize and improve a preliminary protocol for removal of bacterial agents and toxins from samples that would undergo DNA analysis. The protocol must result in samples that are free of infectious or toxic material, but still generate DNA of adequate quantity and quality to meet RCMP standards for identification based on DNA.

In order to obtain and identify possible criminals from DNA evidence, human samples must undergo various steps to isolate and analyse the DNA in a forensic laboratory. These steps consist of (1) the isolation of DNA from samples collected at the crime scene, (2) quantification of the DNA (3) amplification of specific regions on the human chromosomes, (4) analysis of the sequence of amplified DNA, and (5) comparison with possible suspects whose DNA has been banked in the National DNA Data Bank (NDDB) or whose DNA has been collected as part of the investigation.

Ce projet de recherche vise à caractériser plus précisément et à améliorer un protocole préliminaire d'élimination des agents bactériens et des toxines dans des échantillons qui feront l'objet d'une analyse d'ADN. Les échantillons obtenus au moyen de ce protocole devraient être exempts de matières infectieuses ou toxiques, mais permettre d'obtenir une qualité et une quantité d'ADN suffisantes pour satisfaire aux normes de la GRC pour l'identification basée sur l'ADN.

Dans le but d'obtenir des preuves basées sur l'ADN et, ainsi, d'identifier d'éventuels criminels, les échantillons humains doivent subir divers procédés visant à isoler et à analyser l'ADN dans un laboratoire judiciaire. Ces procédés comprennent 1) l'isolement de l'ADN à partir d'échantillons recueillis sur le lieu d'un crime; 2) la quantification de l'ADN; 3) l'amplification de régions spécifiques des chromosomes humains; 4) l'analyse de la séquence de

l'ADN amplifié; 5) la comparaison avec des suspects possibles dont le profil d'ADN est conservé dans la Banque nationale de données génétiques ou dont l'ADN a été recueilli dans le cadre d'une enquête.

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Forensic Analysis; Bioweapons, DNA; criminal investigation